



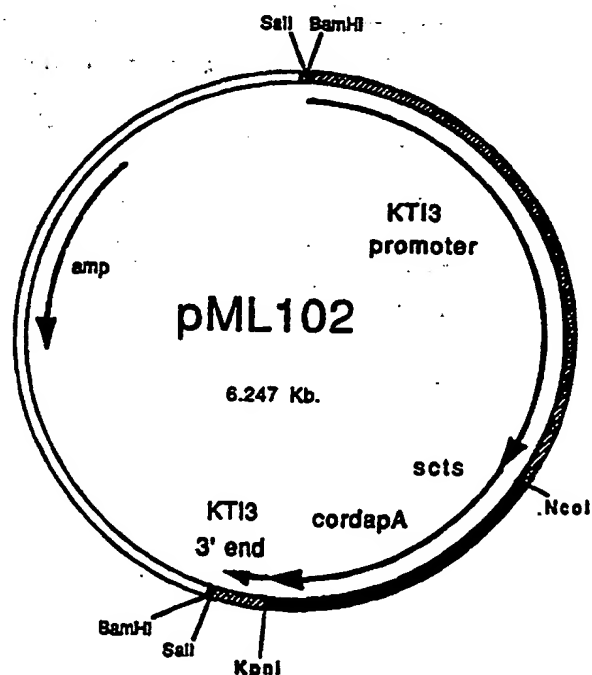
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(54) Title: CHIMERIC GENES AND METHODS FOR INCREASING THE LYSINE CONTENT OF THE SEEDS OF CORN, SOYBEAN AND RAPESEED PLANTS

## (57) Abstract

This invention relates to three chimeric genes, the first encoding dihydrodipicolinic acid synthase (DHDPS), which is insensitive to inhibition by lysine and operably linked to a plant chloroplast transit sequence, a second encoding a lysine-rich protein, and a third encoding a plant lysine ketoglutarate reductase, all operably linked to plant seed-specific regulatory sequences. Methods for their use to produce increased levels of lysine in the seeds of transformed plants are provided. Also provided are transformed corn, rapeseed and soybean plants wherein the seeds accumulate lysine to higher levels than untransformed plants.



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TITLE

CHIMERIC GENES AND METHODS FOR  
INCREASING THE LYSINE CONTENT OF THE  
SEEDS OF CORN, SOYBEAN AND RAPESEED PLANTS

TECHNICAL FIELD

This invention relates to three chimeric genes, the first encoding dihydrodipicolinic acid synthase (DHDPS), which is insensitive to inhibition by lysine and operably linked to a plant chloroplast transit sequence, a second encoding a lysine-rich protein, and a third encoding a plant lysine ketoglutarate reductase, all operably linked to plant seed-specific regulatory sequences. Methods for their use to produce increased levels of lysine in the seeds of transformed plants are provided. Also provided are transformed corn, rapeseed and soybean plants wherein the seeds accumulate lysine to higher levels than untransformed plants.

BACKGROUND OF THE INVENTION

Human food and animal feed derived from many grains are deficient in some of the ten essential amino acids which are required in the animal diet. In corn (*Zea mays* L.), lysine is the most limiting amino acid for the dietary requirements of many animals. Meal derived from other crop plants, e.g., soybean (*Glycine max* L.) or Canola (*Brassica napus*), is used as an additive to corn based animal feeds to supplement this lysine deficiency. Also, additional lysine, produced via fermentation of microbes, is used as a supplement in animal feeds. An increase in the lysine content of meal derived from plant sources would reduce or eliminate the need to supplement mixed grain feeds with microbially produced lysine.

The amino acid content of seeds is determined primarily (90-99%) by the amino acid composition of the proteins in the seed and to a lesser extent (1-10%) by

the free amino acid pools. The quantity of total protein in seeds varies from about 10% of the dry weight in cereals to 20-40% of the dry weight of legumes. Much of the protein-bound amino acids is contained in the seed storage proteins which are synthesized during seed development and which serve as a major nutrient reserve following germination. In many seeds the storage proteins account for 50% or more of the total protein.

To improve the amino acid composition of seeds genetic engineering technology is being used to isolate, and express genes for storage proteins in transgenic plants. For example, a gene from Brazil nut for a seed 2S albumin composed of 26% sulfur-containing amino acids has been isolated [Altenbach et al. (1987) Plant Mol. Biol. 8:239-250] and expressed in the seeds of transformed tobacco under the control of the regulatory sequences from a bean phaseolin storage protein gene. The accumulation of the sulfur-rich protein in the tobacco seeds resulted in an up to 30% increase in the level of methionine in the seeds [Altenbach et al. (1989) Plant Mol. Biol. 13:513-522]. However, no plant seed storage proteins similarly enriched in lysine relative to average lysine content of plant proteins have been identified to date, preventing this approach from being used to increase lysine.

An alternative approach is to increase the production and accumulation of lysine via genetic engineering technology. Lysine, along with threonine, methionine and isoleucine, are amino acids derived from aspartate, and regulation of the biosynthesis of each member of this family is complex, interconnected, and not well understood, especially in plants. Regulation of the metabolic flow in the pathway appears to be primarily via end products in plants. The aspartate family pathway is also regulated at the branch-point



reactions. For lysine this is the condensation of aspartyl  $\beta$ -semialdehyde with pyruvate catalyzed by dihydrodipicolinic acid synthase (DHDPS).

The *E. coli* dapA gene encodes a DHDPS enzyme that  
5 is about 20-fold less sensitive to inhibition by lysine than than a typical plant DHDPS enzyme, e.g., wheat germ DHDPS. The *E. coli* dapA gene has been linked to the 35S promoter of Cauliflower Mosaic Virus and a plant chloroplast transit sequence. The chimeric gene was  
10 introduced into tobacco cells via transformation and shown to cause a substantial increase in free lysine levels in leaves [Glassman et al. (1989) PCT Patent Appl. PCT/US89/01309, Shaul et al. (1992) Plant Jour. 2:203-209, Galili et al. (1992) EPO Patent Appl. 91119328.2, Falco, PCT/US93/02480 (International  
15 Publication Number WO 93/19190). However, the lysine content of the seeds was not increased in any of the transformed plants described in these studies. The same chimeric gene was also introduced into potato cells and  
20 lead to small increases in free lysine in leaves, roots and tubers of regenerated plants [Galili et al. (1992) EPO Patent Appl. 91119328.2, Perl et al. (1992) Plant Mol. Biol. 19:815-823].

Falco, PCT/US93/02480 (International Publication  
25 Number WO 93/19190, linked the *E. coli* dapA gene to the bean phaseolin promoter and a plant chloroplast transit sequence to increase expression in seeds, but still observed no increase in the lysine level in seeds. As noted above, the first step in the lysine biosynthetic  
30 pathway is catalyzed by aspartokinase (AK), and this enzyme has been found to be an important target for regulation in many organisms. Falco isolated a mutant of the *E. coli* lysC gene, which encoded a lysine-feedback-insensitive AK, and linked it to the bean  
35 phaseolin promoter and a plant chloroplast transit

sequence. Expression of this chimeric gene in the seeds of transformed tobacco lead to a substantial increase in the level of threonine, but not lysine. Galili et al. (1992) EPO Patent Appl. 91119328.2 suggest that

5 transforming plants with chimeric genes linking seed-specific promoters to a plant chloroplast transit sequence/*E. coli* dapA gene and plant chloroplast transit sequence/mutant *E. coli* lysC gene will lead to increased lysine levels in seeds. Falco, PCT/US93/02480

10 (International Publication Number WO 93/19190) carried out this experiment by transforming tobacco with a construct containing both the chimeric genes, bean phaseolin promoter/plant chloroplast transit sequence/*E. coli* dapA gene and bean phaseolin

15 promoter/plant chloroplast transit sequence/mutant *E. coli* lysC gene. Simultaneous expression of both genes had no significant effect on the lysine content of the seeds. However, it was noted that a breakdown product of lysine,  $\alpha$ -amino adipic acid, built up in the

20 seeds. This suggested that the accumulation of free lysine in seeds was prevented because of lysine catabolism. In an effort to increase the rate of biosynthesis of lysine, Falco, PCT/US93/02480

25 (International Publication Number WO 93/19190, isolated the *Corynebacterium glutamicum* dapA gene which encodes a completely lysine insensitive DHDPS enzyme. Falco transformed tobacco with a construct containing the chimeric gene, bean phaseolin promoter/plant chloroplast transit sequence/*Corynebacterium glutamicum* dapA gene

30 linked to bean phaseolin promoter/plant chloroplast transit sequence/mutant *E. coli* lysC gene. Simultaneous expression of both these lysine-insensitive enzymes still had no significant effect on the lysine content of the seeds.

Thus, it is clear that the limited understanding of the details of the regulation of the lysine biosynthetic pathway in plants, particularly in seeds, makes the application of genetic engineering technology to  
5 increase lysine content uncertain. It is not known, for most plants, whether lysine is synthesized in seeds or transported to the seeds from leaves. In addition, little is known about storage or catabolism of lysine in seeds. Because free amino acids make up only a small  
10 fraction of the total amino acid content of seeds, over-accumulation must be many-fold in order to significantly affect the total amino acid composition of the seeds. In addition, the effects of over-accumulation of a free amino acid such as lysine on seed development and  
15 viability is not known.

No method to increase the lysine content of seeds via genetic engineering and no examples of seeds having increased lysine levels obtained via genetic engineering were known before the invention described herein.

#### 20 SUMMARY OF THE INVENTION

This invention concerns a novel chimeric gene, and plants transformed using said novel gene, wherein a nucleic acid fragment encoding dihydrodipicolinic acid synthase, which is insensitive to inhibition by lysine,  
25 is operably linked to a plant chloroplast transit sequence and to a plant seed-specific regulatory sequence. In a preferred embodiment, the nucleic acid fragment encoding dihydrodipicolinic acid synthase comprises the nucleotide sequence shown in SEQ ID NO:3:  
30 encoding dihydrodipicolinic acid synthase from Corynebacterium glutamicum. In especially preferred embodiments, the plant chloroplast transit sequence is derived from a gene encoding the small subunit of ribulose 1,5-bisphosphate carboxylase, and the seed-  
35 specific regulatory sequence is from the gene encoding

the  $\beta$  subunit of the seed storage protein phaseolin from the bean Phaseolus vulgaris, the Kunitz trypsin inhibitor 3 gene of Glycine max, or a monocot embryo-specific promoter, preferably from the globulin 1 gene from Zea maize.

The genes described may be used, for example, for transforming plants, preferably corn, rapeseed or soybean plants. Also claimed are seeds obtained from the transformed plants. The invention can produce transformed plants wherein the seeds of the plants accumulate lysine to a level at least ten percent higher than in seeds of untransformed plants, preferably ten to four hundred percent higher than in untransformed plants.

The invention further concerns a method for obtaining a plant, preferably a corn, rapeseed or soybean plant wherein the seeds of the plants accumulate lysine to a level from ten percent to four hundred percent higher than seeds of untransformed plants comprising:

- (a) transforming plant cells, preferably corn, rapeseed or soybean cells, with the chimeric gene described above;
- (b) regenerating fertile mature plants from the transformed plant cells obtained from step (a) under conditions suitable to obtain seeds;
- (c) screening the progeny seed of step (b) for lysine content; and
- (d) selecting those lines whose seeds contain increased levels of lysine. Transformed plants obtained from this method are also claimed.

The invention additionally concerns a nucleic acid fragment comprising

- (a) a first chimeric gene described above and

(b) a second chimeric gene wherein a nucleic acid fragment encoding a lysine-rich protein, wherein the weight percent lysine is at least 15%, is operably linked to a plant seed-specific regulatory sequence.

5 Also described is a nucleic acid fragment comprising

(a) a first chimeric gene as described above and

(b) a second chimeric gene wherein a nucleic acid fragment encoding a lysine-rich protein comprises a  
10 nucleic acid sequence encoding a protein comprising n heptad units (d e f g a b c), each heptad being either the same or different, wherein:

n is at least 4;

15 a and d are independently selected from the group consisting of Met, Leu, Val, Ile and Thr;

e and g are independently selected from the group consisting of the acid/base  
20 pairs Glu/Lys, Lys/Glu, Arg/Glu, Arg/Asp, Lys/Asp, Glu/Arg, Asp/Arg and Asp/Lys; and

b, c and f are independently any amino acids except Gly or Pro and at least  
25 two amino acids of b, c and f in each heptad are selected from the group consisting of Glu, Lys, Asp, Arg, His, Thr, Ser, Asn, Ala, Gln and Cys,

said nucleic acid fragment is operably linked to a plant  
30 seed-specific regulatory sequence.

Further described herein is a nucleic acid fragment comprising

(a) a first chimeric gene described above; and

(b) a second chimeric gene wherein a nucleic  
35 acid fragment encoding a lysine-rich protein comprises a

nucleic acid sequence encoding a protein having the amino acid sequence (MEEKLKA)<sub>6</sub>(MEEKMKA)<sub>2</sub> is operably linked to a plant seed-specific regulatory sequence.

Also claimed herein are plants containing various  
5 embodiments of the described first chimeric genes and second chimeric genes and the described nucleic acid fragments and seeds obtained from such plants.

The invention further concerns a nucleic acid fragment comprising

10 (a) a first chimeric gene as described above and

(b) a second chimeric gene wherein a nucleic acid fragment encoding a lysine ketoglutarate reductase is operably linked in the sense or antisense orientation  
15 to a plant seed-specific regulatory sequence. Also claimed is a plant comprising in its genome that nucleic acid fragment and a seed obtained from such plant.

#### BRIEF DESCRIPTION OF THE

#### DRAWINGS AND SEQUENCE DESCRIPTIONS

20 The invention can be more fully understood from the following detailed description and the accompanying drawings and the sequence descriptions which form a part of this application.

Figure 1 shows an alpha helix from the side and top  
25 views.

Figure 2 shows end (Figure 2a) and side (Figure 2b) views of an alpha helical coiled-coil structure.

Figure 3 shows the chemical structure of leucine and methionine emphasizing their similar shapes.

30 Figure 4 shows a schematic representation of a seed-specific gene expression cassette.

Figure 5A shows a map of the binary plasmid vector pZS199; Figure 5B shows a map of the binary plasmid vector pFS926.

Figure 6A shows a map of the plasmid vector pBT603; Figure 6B shows a map of the plasmid vector pBT614.

Figure 7 depicts the strategy for creating a vector (pSK5) for use in construction and expression of the SSP  
5 gene sequences.

Figure 8 shows the strategy for inserting oligo-nucleotide sequences into the unique Ear I site of the base gene sequence.

Figure 9 shows the insertion of the base gene  
10 oligonucleotides into the Nco I/EcoR I sites of pSK5 to create the plasmid pSK6. This base gene sequence was used as in Figure 8 to insert the various SSP coding regions at the unique Ear I site to create the cloned segments listed.

Figure 10 shows the insertion of the 63 bp  
15 "segment" oligonucleotides used to create non-repetitive gene sequences for use in the duplication scheme in Figure 11.

Figure 11 (A and B) shows the strategy for  
20 multiplying non-repetitive gene "segments" utilizing in-frame fusions.

Figure 12 shows the vectors containing seed specific promoter and 3' sequence cassettes. SSP sequences were inserted into these vectors using the  
25 Nco I and Asp718 sites.

Figure 13 shows a map of the binary plasmid vector pZS97.

Figure 14 shows a map of the plasmid vector pML63.

Figure 15 shows a map of the plasmid vector pML102  
30 carrying a chimeric gene wherein seed specific regulatory sequences (from the soybean Kunitz trypsin inhibitor 3 gene) are linked to a chloroplast transit sequence (from the small subunit of soybean ribulose bis-phosphate carboxylase) and the coding sequence for

lysine-insensitive dihydrodipicolinic acid synthase (the dapA gene from Corynebacterium glutamicum).

SEQ ID NOS:1 and 2 were used in Example 1 as PCR primers for the isolation of the Corynebacterium dapA gene.

SEQ ID NO:3 shows the nucleotide and amino acid sequence of the coding region of the wild type Corynebacterium dapA gene, which encodes lysine-insensitive DHDPs, described in Example 1.

SEQ ID NO:4 shows an oligonucleotide used in Example 2 to create an Nco I site at the translation start codon of the E. coli dapA gene.

SEQ ID NO:5 shows the nucleotide and amino acid sequence of the coding region of the wild type E. coli lysC gene, which encodes AKIII, described in Example 3.

SEQ ID NOS:6 and 7 were used in Example 3 to create an Nco I site at the translation start codon of the E. coli lysC gene.

SEQ ID NOS:8, 9, 10 and 11 were used in Example 4 to create a chloroplast transit sequence and link the sequence to the E. coli lysC-M4, E. coli dapA and Corynebacteria dapA genes.

SEQ ID NOS:12 and 13 were used in Example 4 to create a Kpn I site immediately following the translation stop codon of the E. coli dapA gene.

SEQ ID NOS:14 and 15 were used in Example 4 as PCR primers to create a soybean chloroplast transit sequence and link the sequence to the Corynebacterium dapA gene.

SEQ ID NOS:16-92 represent nucleic acid fragments and the polypeptides they encode that are used to create chimeric genes for lysine-rich synthetic seed storage proteins suitable for expression in the seeds of plants.

SEQ ID NOS:93-98 were used in Example 12 to create a corn chloroplast transit sequence.



SEQ ID NOS:99 and 100 were used in Example 12 as PCR primers to create a corn chloroplast transit sequence and link the sequence to the *E. coli* *dapA* gene.

The Sequence Descriptions contain the one letter  
5 code for nucleotide sequence characters and the three  
letter codes for amino acids as defined in conformity  
with the IUPAC-IYUB standards described in Nucleic Acids  
Research 13:3021-3030(1985) and in the Biochemical  
Journal 219 (No. 2):345-373(1984), which are  
10 incorporated by reference herein.

#### DETAILED DESCRIPTION OF THE INVENTION:

The teachings below describe nucleic acid fragments  
and procedures useful for increasing the accumulation of  
lysine in the seeds of transformed plants, as compared  
15 to levels of lysine in untransformed plants. In order  
to increase the accumulation of free lysine in the seeds  
of plants via genetic engineering, a determination was  
made of which enzymes in this pathway controlled the  
pathway in the seeds of plants. In order to accomplish  
20 this, genes encoding enzymes in the pathway were  
isolated from bacteria. Intracellular localization  
sequences and suitable regulatory sequences for  
expression in the seeds of plants were linked to create  
chimeric genes. The chimeric genes were then introduced  
25 into plants via transformation and assessed for their  
ability to elicit accumulation of the lysine in seeds.  
Expression of lysine-insensitive dihydrodipicolinic acid  
synthase (DHDPS), under control of a strong seed-  
specific promoter, is shown to increase free lysine  
30 levels 10 to 100 fold in corn, rapeseed and soybean  
seeds.

It has been discovered that the full potential for  
accumulation of excess free lysine in seeds is reduced  
by lysine catabolism. Provided herein are two  
35 alternative routes to prevent the loss of excess lysine

due to catabolism. In the first approach, lysine catabolism is prevented through reduction in the activity of the enzyme lysine ketoglutarate reductase (LKR), which catalyzes the first step in lysine breakdown. A procedure to isolate plant LKR genes is provided. Chimeric genes for expression of antisense LKR RNA or for cosuppression of LKR in the seeds of plants are created. The chimeric gene is then linked to the chimeric DHDPS gene and both are introduced into plants via transformation simultaneously, or the genes are brought together by crossing plants transformed independently with each of the chimeric genes.

In the second approach, excess free lysine is incorporated into a form that is insensitive to breakdown, e.g., by incorporating it into a di-, tri- or oligopeptide, or a lysine-rich storage protein. The design of polypeptides which can be expressed *in vivo* to serve as lysine-rich seed storage proteins is provided. Genes encoding the lysine-rich synthetic storage proteins (SSP) are synthesized and chimeric genes wherein the SSP genes are linked to suitable regulatory sequences for expression in the seeds of plants are created. The SSP chimeric gene is then linked to the chimeric DHDPS gene and both are introduced into plants via transformation simultaneously, or the genes are brought together by crossing plants transformed independently with each of the chimeric genes.

A method for transforming plants, preferably corn, rapeseed and soybean plants is taught herein wherein the resulting seeds of the plants have at least ten percent, preferably ten percent to 400 percent greater lysine than the seeds of untransformed plants. Provided as examples herein are transformed rapeseed plants with seed lysine levels increased by 100% over untransformed plants, soybean plants with seed lysine levels increased

by 400% over untransformed plants, and transformed corn plants with seed lysine levels increased by 130% over untransformed plants.

In the context of this disclosure, a number of terms are utilized. As used herein, the term "nucleic acid" refers to a large molecule which can be single-stranded or double-stranded, composed of monomers (nucleotides) containing a sugar, phosphate and either a purine or pyrimidine. A "nucleic acid fragment" is a fraction of a given nucleic acid molecule. In higher plants, deoxyribonucleic acid (DNA) is the genetic material while ribonucleic acid (RNA) is involved in the transfer of the information in DNA into proteins. A "genome" is the entire body of genetic material contained in each cell of an organism. The term "nucleotide sequence" refers to a polymer of DNA or RNA which can be single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases capable of incorporation into DNA or RNA polymers. "Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding) and following (3' non-coding) the coding region. "Native" gene refers to the gene as found in nature with its own regulatory sequences. "Chimeric" gene refers to a gene comprising heterogeneous regulatory and coding sequences. "Endogenous" gene refers to the native gene normally found in its natural location in the genome. A "foreign" gene refers to a gene not normally found in the host organism but that is introduced by gene transfer.

"Coding sequence" refers to a DNA sequence that codes for a specific protein and excludes the non-coding sequences.

"Initiation codon" and "termination codon" refer to a unit of three adjacent nucleotides in a coding sequence that specifies initiation and chain termination, respectively, of protein synthesis (mRNA translation). "Open reading frame" refers to the amino acid sequence encoded between translation initiation and termination codons of a coding sequence.

As used herein, suitable "regulatory sequences" refer to nucleotide sequences located upstream (5'), within, and/or downstream (3') to a coding sequence, which control the transcription and/or expression of the coding sequences, potentially in conjunction with the protein biosynthetic apparatus of the cell. These regulatory sequences include promoters, translation leader sequences, transcription termination sequences, and polyadenylation sequences.

"Promoter" refers to a DNA sequence in a gene, usually upstream (5') to its coding sequence, which controls the expression of the coding sequence by providing the recognition for RNA polymerase and other factors required for proper transcription. A promoter may also contain DNA sequences that are involved in the binding of protein factors which control the effectiveness of transcription initiation in response to physiological or developmental conditions. It may also contain enhancer elements.

An "enhancer" is a DNA sequence which can stimulate promoter activity. It may be an innate element of the promoter or a heterologous element inserted to enhance the level and/or tissue-specificity of a promoter.

"Constitutive promoters" refers to those that direct gene expression in all tissues and at all times.

"Organ-specific" or "development-specific" promoters as referred to herein are those that direct gene expression almost exclusively in specific organs, such as leaves or

seeds, or at specific development stages in an organ, such as in early or late embryogenesis, respectively.

The term "operably linked" refers to nucleic acid sequences on a single nucleic acid molecule which are associated so that the function of one is affected by the other. For example, a promoter is operably linked with a structure gene when it is capable of affecting the expression of that structural gene (i.e., that the structural gene is under the transcriptional control of the promoter).

The term "expression", as used herein, is intended to mean the production of the protein product encoded by a gene. More particularly, "expression" refers to the transcription and stable accumulation of the sense (mRNA) or the antisense RNA derived from the nucleic acid fragment(s) of the invention that, in conjunction with the protein apparatus of the cell, results in altered levels of protein product. "Antisense inhibition" refers to the production of antisense RNA transcripts capable of preventing the expression of the target protein. "Overexpression" refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal or non-transformed organisms. "Cosuppression" refers to the expression of a foreign gene which has substantial homology to an endogenous gene resulting in the suppression of expression of both the foreign and the endogenous gene. "Altered levels" refers to the production of gene product(s) in transgenic organisms in amounts or proportions that differ from that of normal or non-transformed organisms.

The "3' non-coding sequences" refers to the DNA sequence portion of a gene that contains a polyadenylation signal and any other regulatory signal capable of affecting mRNA processing or gene expression.

The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor.

The "translation leader sequence" refers to that DNA sequence portion of a gene between the promoter and coding sequence that is transcribed into RNA and is present in the fully processed mRNA upstream (5') of the translation start codon. The translation leader sequence may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency.

"Mature" protein refers to a post-translationally processed polypeptide without its targeting signal. "Precursor" protein refers to the primary product of translation of mRNA. A "chloroplast targeting signal" is an amino acid sequence which is translated in conjunction with a protein and directs it to the chloroplast. "Chloroplast transit sequence" refers to a nucleotide sequence that encodes a chloroplast targeting signal.

"Transformation" herein refers to the transfer of a foreign gene into the genome of a host organism and its genetically stable inheritance. Examples of methods of plant transformation include Agrobacterium-mediated transformation and particle-accelerated or "gene gun" transformation technology.

"Amino acids" herein refer to the naturally occurring L amino acids (Alanine, Arginine, Aspartic acid, Asparagine, Cystine, Glutamic acid, Glutamine, Glycine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Proline, Phenylalanine, Serine, Threonine, Tryptophan, Tyrosine, and Valine). "Essential amino acids" are those amino acids which cannot be synthesized by animals. A "polypeptide" or "protein" as used herein refers to a molecule composed of monomers (amino acids)

linearly linked by amide bonds (also known as peptide bonds).

"Synthetic protein" herein refers to a protein consisting of amino acid sequences that are not known to occur in nature. The amino acid sequence may be derived from a consensus of naturally occurring proteins or may be entirely novel.

"Primary sequence" refers to the connectivity order of amino acids in a polypeptide chain without regard to the conformation of the molecule. Primary sequences are written from the amino terminus to the carboxy terminus of the polypeptide chain by convention.

"Secondary structure" herein refers to physico-chemically favored regular backbone arrangements of a polypeptide chain without regard to variations in side chain identities or conformations. "Alpha helices" as used herein refer to right-handed helices with approximately 3.6 residues per turn of the helix. An "amphipathic helix" refers herein to a polypeptide in a helical conformation where one side of the helix is predominantly hydrophobic and the other side is predominantly hydrophilic.

"Coiled-coil" herein refers to an aggregate of two parallel right-handed alpha helices which are wound around each other to form a left-handed superhelix.

"Salt bridges" as discussed here refer to acid-base pairs of charged amino acid side chains so arranged in space that an attractive electrostatic interaction is maintained between two parts of a polypeptide chain or between one chain and another.

"Host cell" means the cell that is transformed with the introduced genetic material.

#### Isolation of DHPS genes

The *E. coli* dapA gene (ecodapA) was obtained as a bacteriophage lambda clone from an ordered library of

3400 overlapping segments of *E. coli* DNA constructed by Kohara, Akiyame and Isono [Kohara et al. (1987) Cell 50:595-508]. Details of the isolation and modification of *ecodapA* are presented in Example 1. The *ecodapA* gene encodes a DHDPS enzyme that is at least 20-fold less sensitive to inhibition by lysine than a typical plant enzyme, e.g., wheat DHDPS. For purposes of the present invention, 20-fold less sensitive to inhibition by lysine is termed lysine-insensitive.

The *Corynebacterium dapA* gene (*cordapA*) was isolated from genomic DNA from ATCC strain 13032 using polymerase chain reaction (PCR). The nucleotide sequence of the *Corynebacterium dapA* gene has been published [Bonnassie et al. (1990) Nucleic Acids Res. 18:6421]. From the sequence it was possible to design oligonucleotide primers for polymerase chain reaction (PCR) that would allow amplification of a DNA fragment containing the gene, and at the same time add unique restriction endonuclease sites at the start codon and just past the stop codon of the gene to facilitate further constructions involving the gene. The details of the isolation of the *Corynebacterium dapA* (*cordapA*) gene are presented in Example 1. The *cordapA* gene encodes a preferred lysine-insensitive DHDPS enzyme that is unaffected by the presence of 70mM lysine in the enzyme reaction mix.

The isolation of other genes encoding DHDPS has been described in the literature. A cDNA encoding DHDPS from wheat [Kaneko et al. (1990) J. Biol. Chem. 265:17451-17455], and a cDNA encoding DHDPS from corn [Frisch et al. (1991) Mol. Gen. Genet. 228:287-293] are two examples of plant DHDPS genes that have been isolated and sequenced. The plant genes encode wild type lysine-sensitive DHDPS enzymes. However, Negrutui et al. [(1984) Theor. Appl. Genet. 68:11-20], obtained



two AEC-resistant tobacco mutants in which DHDPS activity was less sensitive to lysine inhibition than the wild type enzyme. This indicates that these tobacco mutants contain DHDPS genes encoding lysine-resistant enzyme. These genes could be readily isolated from the tobacco mutants using the methods already described for isolating the wheat or corn genes or, alternatively, by using the wheat or corn genes as heterologous hybridization probes.

Still other genes encoding DHDPS can be isolated by using either the *E. coli* dapA gene, the cordapA gene, or either of the plant DHDPS genes as DNA hybridization probes. Alternatively, other genes encoding DHDPS could be isolated by functional complementation of an *E. coli* dapA mutant, as was done to isolate the cordapA gene [Yeh et al. (1988) Mol. Gen. Genet. 212:105-111] and the corn DHDPS gene.

#### Construction of Chimeric Genes for Expression of dapA Coding Region in Plants

The expression of foreign genes in plants is well-established [De Blaere et al. (1987) Meth. Enzymol. 143:277-291]. Proper level of expression of dapA mRNA may require the use of different chimeric genes utilizing different promoters. Such chimeric genes can be transferred into host plants either together in a single expression vector or sequentially using more than one vector. A preferred class of heterologous hosts for the expression of the coding sequence of the dapA genes are eukaryotic hosts, particularly the cells of higher plants. Particularly preferred among the higher plants and the seeds derived from them are rapeseed (Brassica napus, B. campestris) and soybean (Glycine max).

The origin of promoter chosen to drive the expression of the coding sequence is not critical as long as it has sufficient transcriptional activity to

accomplish the invention by expressing translatable mRNA for dapA genes in the desired host tissue. Preferred promoters are those that allow expression of the protein specifically in seeds. This may be especially useful, since seeds are the primary source of vegetable amino acids and also since seed-specific expression will avoid any potential deleterious effect in non-seed organs. Examples of seed-specific promoters include, but are not limited to, the promoters of seed storage proteins. The seed storage proteins are strictly regulated, being expressed almost exclusively in seeds in a highly organ-specific and stage-specific manner [Higgins et al. (1984) Ann. Rev. Plant Physiol. 35:191-221; Goldberg et al. (1989) Cell 56:149-160; Thompson et al. (1989) BioEssays 10:108-113]. Moreover, different seed storage proteins may be expressed at different stages of seed development.

There are currently numerous examples for seed-specific expression of seed storage protein genes in transgenic dicotyledonous plants. These include genes from dicotyledonous plants for bean  $\beta$ -phaseolin [Sengupta-Goplalan et al. (1985) Proc. Natl. Acad. Sci. USA 82:3320-3324; Hoffman et al. (1988) Plant Mol. Biol. 11:717-729], bean lectin [Voelker et al. (1987) EMBO J. 6: 3571-3577], soybean lectin [Okamuro et al. (1986) Proc. Natl. Acad. Sci. USA 83:8240-8244], soybean kunitz trypsin inhibitor [Perez-Grau et al. (1989) Plant Cell 1:095-1109], soybean  $\beta$ -conglycinin [Beachy et al. (1985) EMBO J. 4:3047-3053; Barker et al. (1988) Proc. Natl. Acad. Sci. USA 85:458-462; Chen et al. (1988) EMBO J. 7:297-302; Chen et al. (1989) Dev. Genet. 10:112-122; Naito et al. (1988) Plant Mol. Biol. 11:109-123], pea vicilin [Higgins et al. (1988) Plant Mol. Biol. 11:683-695], pea convicilin [Newbigin et al. (1990) Planta 180:461], pea legumin [Shirsat et al. (1989) Mol.

Gen. Genetics 215:326]; rapeseed napin [Radke et al. (1988) Theor. Appl. Genet. 75:685-694] as well as genes from monocotyledonous plants such as for maize 15 kD zein [Hoffman et al. (1987) EMBO J. 6:3213-3221; 5 Schernthaner et al. (1988) EMBO J. 7:1249-1253; Williamson et al. (1988) Plant Physiol. 88:1002-1007], barley  $\beta$ -hordein [Marris et al. (1988) Plant Mol. Biol. 10:359-366] and wheat glutenin [Colot et al. (1987) EMBO J. 6:3559-3564]. Moreover, promoters of seed-specific 10 genes, operably linked to heterologous coding sequences in chimeric gene constructs, also maintain their temporal and spatial expression pattern in transgenic plants. Such examples include Arabidopsis thaliana 2S seed storage protein gene promoter to express enkephalin 15 peptides in Arabidopsis and B. napus seeds [Vandekerckhove et al. (1989) Bio/Technology 7:929-932], bean lectin and bean  $\beta$ -phaseolin promoters to express luciferase [Riggs et al. (1989) Plant Sci. 63:47-57], and wheat glutenin promoters to express chloramphenicol 20 acetyl transferase [Colot et al. (1987) EMBO J. 6:3559-3564].

Of particular use in the expression of the nucleic acid fragment of the invention will be the promoters from several extensively-characterized seed storage 25 protein genes such as those for bean  $\beta$ -phaseolin [Sengupta-Goplalan et al. (1985) Proc. Natl. Acad. Sci. USA 82:3320-3324; Hoffman et al. (1988) Plant Mol. Biol. 11:717-729], soybean Kunitz trypsin inhibitor [Jofuku et al. (1989) Plant Cell 1:1079-1093; Perez-Grau et al. 30 (1989) Plant Cell 1:1095-1109], soybean  $\beta$ -conglycinin [Harada et al. (1989) Plant Cell 1:415-425], and rapeseed napin [Radke et al. (1988) Theor. Appl. Genet. 75:685-694]. Promoters of genes for bean  $\beta$ -phaseolin and soybean  $\beta$ -conglycinin storage protein will be

particularly useful in expressing the dapA mRNA in the cotyledons at mid- to late-stages of seed development.

Also of particular use in the expression of the nucleic acid fragments of the invention will be the  
5 heterologous promoters from several extensively characterized corn seed storage protein genes such as endosperm-specific promoters from the 10 kD zein [Kirihara et al. (1988) Gene 71:359-370], the 27 kD zein [Prat et al. (1987) Gene 52:51-49; Gallardo et al.  
10 (1988) Plant Sci. 54:211-281; Reina et al. (1990) Nucleic Acids Res. 18:6426-6426], and the 19 kD zein [Marks et al. (1985) J. Biol. Chem. 260:16451-16459]. The relative transcriptional activities of these promoters in corn have been reported [Kodrzyck et al.  
15 (1989) Plant Cell 1:105-114] providing a basis for choosing a promoter for use in chimeric gene constructs for corn. For expression in corn embryos, the strong, embryo-specific promoter from the globulin-1 (GLB1) gene [Kriz (1989) Biochemical Genetics 27:239-251, Wallace et al.  
20 al. (1991) Plant Physiol. 95:973-975] can be used.

It is envisioned that the introduction of enhancers or enhancer-like elements into other promoter constructs will also provide increased levels of primary transcription for dapA genes to accomplish the  
25 invention. These would include viral enhancers such as that found in the 35S promoter [Odell et al. (1988) Plant Mol. Biol. 10:263-272], enhancers from the opine genes [Fromm et al. (1989) Plant Cell 1:977-984], or enhancers from any other source that result in increased  
30 transcription when placed into a promoter operably linked to the nucleic acid fragment of the invention.

Of particular importance is the DNA sequence element isolated from the gene for the  $\alpha'$ -subunit of  $\beta$ -conglycinin that can confer 40-fold seed-specific  
35 enhancement to a constitutive promoter [Chen et al.

(1988) EMBO J. 7:297-302; Chen et al. (1989) Dev. Genet. 10:112-122]. One skilled in the art can readily isolate this element and insert it within the promoter region of any gene in order to obtain seed-specific enhanced  
5 expression with the promoter in transgenic plants. Insertion of such an element in any seed-specific gene that is expressed at different times than the  $\beta$ -conglycinin gene will result in expression in transgenic plants for a longer period during seed  
10 development.

Any 3' non-coding region capable of providing a polyadenylation signal and other regulatory sequences that may be required for the proper expression of the dapA coding regions can be used to accomplish the  
15 invention. This would include the 3' end from any storage protein such as the 3' end of the bean phaseolin gene, the 3' end of the soybean  $\beta$ -conglycinin gene, the 3' end from viral genes such as the 3' end of the 35S or the 19S cauliflower mosaic virus transcripts, the 3' end  
20 from the opine synthesis genes, the 3' ends of ribulose 1,5-bisphosphate carboxylase or chlorophyll a/b binding protein, or 3' end sequences from any source such that the sequence employed provides the necessary regulatory information within its nucleic acid sequence to result  
25 in the proper expression of the promoter/coding region combination to which it is operably linked. There are numerous examples in the art that teach the usefulness of different 3' non-coding regions [for example, see Ingelbrecht et al. (1989) Plant Cell 1:671-680].

30 DNA sequences coding for intracellular localization sequences may be added to the dapA coding sequence if required for the proper expression of the proteins to accomplish the invention. Plant amino acid biosynthetic enzymes are known to be localized in the chloroplasts  
35 and therefore are synthesized with a chloroplast

targeting signal. Bacterial proteins such as Corynebacterium DHDPS have no such signal. A chloroplast transit sequence could, therefore, be fused to the dapA coding sequence. Preferred chloroplast transit sequences are those of the small subunit of ribulose 1,5-bisphosphate carboxylase, e.g. from soybean [Berry-Lowe et al. (1982) J. Mol. Appl. Genet. 1:483-498] for use in dicotyledonous plants and from corn [Lebrun et al. (1987) Nucleic Acids Res. 15:4360] for use in monocotyledonous plants.

#### Introduction of dapA

#### Chimeric Genes into Plants

Various methods of introducing a DNA sequence (i.e., of transforming) into eukaryotic cells of higher plants are available (see EPO publications 0 295 959 A2 and 0 138 341 A1). Such methods include those based on transformation vectors based on the Ti and Ri plasmids of Agrobacterium spp. It is particularly preferred to use the binary type of these vectors. Ti-derived vectors transform a wide variety of higher plants, including monocotyledonous and dicotyledonous plants, such as soybean, cotton and rape [Pacciotti et al. (1985) Bio/Technology 3:241; Byrne et al. (1987) Plant Cell, Tissue and Organ Culture 8:3; Sukhapinda et al. (1987) Plant Mol. Biol. 8:209-216; Lorz et al. (1985) Mol. Gen. Genet. 199:178; Potrykus (1985) Mol. Gen. Genet. 199:183].

For introduction into plants the chimeric genes of the invention can be inserted into binary vectors as described in Examples 6-12. The vectors are part of a binary Ti plasmid vector system [Bevan, (1984) Nucl. Acids. Res. 12:8711-8720] of Agrobacterium tumefaciens.

Other transformation methods are available to those skilled in the art, such as direct uptake of foreign DNA constructs [see EPO publication 0 295 959 A2],

techniques of electroporation [see Fromm et al. (1986) Nature (London) 319:791] or high-velocity ballistic bombardment with metal particles coated with the nucleic acid constructs [see Kline et al. (1987) Nature (London) 327:70, and see U.S. Pat. No. 4,945,050]. Once transformed, the cells can be regenerated by those skilled in the art.

Of particular relevance are the recently described methods to transform foreign genes into commercially important crops, such as rapeseed [see De Block et al. (1989) Plant Physiol. 91:694-701], sunflower [Everett et al. (1987) Bio/Technology 5:1201], soybean [McCabe et al. (1988) Bio/Technology 6:923; Hinchee et al. (1988) Bio/Technology 6:915; Chee et al. (1989) Plant Physiol. 91:1212-1218; Christou et al. (1989) Proc. Natl. Acad. Sci USA 86:7500-7504; EPO Publication 0 301 749 A2], and corn [Gordon-Kamm et al. (1990) Plant Cell 2:603-618; Fromm et al. (1990) Biotechnology 8:833-839].

For introduction into plants by high-velocity ballistic bombardment, the chimeric genes of the invention can be inserted into suitable vectors as described in Example 6.

#### Expression of dapA Chimeric Genes in Rapeseed, Soybean and Corn Plants

To analyze for expression of the chimeric dapA gene in seeds and for the consequences of expression on the amino acid content in the seeds, a seed meal can be prepared as described in Examples 5 or 6 or by any other suitable method. The seed meal can be partially or completely defatted, via hexane extraction for example, if desired. Protein extracts can be prepared from the meal and analyzed for DHDPS enzyme activity. Alternatively the presence of the DHDPS protein can be tested for immunologically by methods well-known to

those skilled in the art. Nearly all of the transformants expressed the foreign DHDPS protein (see Examples 5, 6 and 13). To measure free amino acid composition of the seeds, free amino acids can be  
5 extracted from the meal and analyzed by methods known to those skilled in the art (see Examples 5 and 6 for suitable procedures).

Rapeseed transformants expressing DHDPS protein showed a greater than 100-fold increase in free lysine  
10 level in their seeds. There was a good correlation between transformants expressing higher levels of DHDPS protein and those having higher levels of free lysine. Among the transformants, there has been no greater accumulation of free lysine due to expression of a  
15 lysine insensitive AK enzyme along with a lysine-insensitive DHDPS compared to expression of a lysine-insensitive DHDPS alone. Thus, in rapeseed, expression of a lysine-insensitive DHDPS in seeds is necessary and sufficient to cause a large increase in free lysine. A  
20 high level of  $\alpha$ -amino adipic acid, indicative of lysine catabolism, was observed in all of the transformed lines with increased levels of free lysine.

To measure the total amino acid composition of mature rapeseed seeds, defatted meal was analyzed as  
25 described in Example 5. Relative amino acid levels in the seeds were compared as percentages of lysine to total amino acids. The highest expressing lines showed a nearly 2-fold increase in the lysine level in the seeds, so that lysine makes up about 12% of the total  
30 seed amino acids.

Twenty-one of twenty-three soybean transformants expressed the DHDPS protein. Analysis of single seeds of these transformants showed excellent correlation between expression of the GUS transformation marker gene  
35 and DHDPS in individual seeds. Therefore, the GUS and



DHDPS genes are integrated at the same site in the soybean genome.

There was excellent correlation between transformants expressing Corynebacteria DHDPS protein and those having higher levels of free lysine. From 20-fold to 120-fold increases in free lysine level was observed in seeds expressing Corynebacteria DHDPS.

Analyses of free lysine levels in individual seeds from transformants in which the transgenes segregated as a single locus revealed that the increase in free lysine level was significantly higher in about one-fourth of the seeds. Since one-fourth of the seeds are expected to be homozygous for the transgene, it is likely that the higher lysine seeds are the homozygotes. Furthermore, this indicates that the level of increase in free lysine is dependent upon the copy number of the DHDPS gene. Therefore, lysine levels could be further increased by making hybrids of two different transformants, and obtaining progeny that are homozygous at both transgene loci, thus increasing the copy number of the DHDPS gene from two to four.

A high level of saccharopine, indicative of lysine catabolism, was observed in seeds that contained high levels of lysine. Thus, prevention of lysine catabolism by inactivation of lysine ketoglutarate reductase should further increase the accumulation of free lysine in the seeds. Alternatively, incorporation of lysine into a peptide or lysine-rich protein would prevent catabolism and lead to an increase in the accumulation of lysine in the seeds.

Total lysine levels were significantly increased in seeds expressing Corynebacteria DHDPS protein. Seeds with a 10-260% increase in the lysine level compared to the untransformed control were observed. Expression of DHDPS along with a lysine-insensitive aspartokinase

enzyme resulted in lysine increases of more than 400%. Thus, these seeds contain much more lysine than any previous soybean seed.

Expression of the Corynebacterium DHDPS protein, driven by either the corn globulin 1 promoter for expression in the embryo or the corn glutelin 2 promoter for expression in the endosperm, was observed in the corn seeds. Free lysine levels in the seeds increased from about 1.4% of free amino acids in control seeds to 15-27% of free amino acids in seeds expressing Corynebacterium DHDPS from the globulin 1 promoter. A smaller increase in free lysine was observed in seeds expressing Corynebacterium DHDPS from the glutelin 2 promoter. Thus to increase lysine, it may be better to express this enzyme in the embryo rather than the endosperm. A high level of saccharopine, indicative of lysine catabolism, was observed in seeds that contained high levels of lysine. The increased accumulation of free lysine in seeds expressing Corynebacterium DHDPS from the globulin 1 promoter was sufficient to result in substantial increases (35%-130%) in the total lysine content of the seeds.

#### Isolation of a Plant

##### Lysine Ketoglutarate Reductase Gene

To accumulate higher levels of free lysine it may be desirable to prevent lysine catabolism. Evidence indicates that lysine is catabolized in plants via the saccharopine pathway. The first enzymatic evidence for the existence of this pathway was the detection of lysine ketoglutarate reductase (LKR) activity in immature endosperm of developing maize seeds [Arruda et al. (1982) Plant Physiol. 69:988-989]. LKR catalyzes the first step in lysine catabolism, the condensation of L-lysine with  $\alpha$ -ketoglutarate into saccharopine using NADPH as a cofactor. LKR activity increases sharply

from the onset of endosperm development in corn, reaches a peak level at about 20 days after pollination, and then declines [Arruda et al. (1983) *Phytochemistry* 22:2687-2689]. In order to prevent the catabolism of lysine it would be desirable to reduce or eliminate LKR expression or activity. This could be accomplished by cloning the LKR gene, preparing a chimeric gene for cosuppression of LKR or preparing a chimeric gene to express antisense RNA for LKR, and introducing the chimeric gene into plants via transformation.

Several methods to clone a plant LKR gene are available to one skilled in the art. The protein can be purified from corn endosperm, as described in Brochetto-Braga et al. [(1992) *Plant Physiol.* 98:1139-1147] and used to raise antibodies. The antibodies can then be used to screen an cDNA expression library for LKR clones. Alternatively the purified protein can be used to determine amino acid sequence at the amino-terminal of the protein or from protease derived internal peptide fragments. Degenerate oligonucleotide probes can be prepared based upon the amino acid sequence and used to screen a plant cDNA or genomic DNA library via hybridization. Another method makes use of an *E. coli* strain that is unable to grow in a synthetic medium containing 20 µg/mL of L-lysine. Expression of LKR full-length cDNA in this strain will reverse the growth inhibition by reducing the lysine concentration. Construction of a suitable *E. coli* strain and its use to select clones from a plant cDNA library that lead to lysine-resistant growth is described in Example 7.

In order to block expression of the LKR gene in transformed plants, a chimeric gene designed for cosuppression of LKR can be constructed by linking the LKR gene or gene fragment to any of the plant promoter sequences described above (U.S. Patent No. 5,231,020).

Alternatively, a chimeric gene designed to express antisense RNA for all or part of the LKR gene can be constructed by linking the LKR gene or gene fragment in reverse orientation to any of the plant promoter sequences described above (Eur. Patent Applic. No. 84112647.7). Either the cosuppression or antisense chimeric gene could be introduced into plants via transformation. Transformants wherein expression of the endogenous LKR gene is reduced or eliminated are selected.

Preferred promoters for the chimeric genes would be seed-specific promoters. For soybean, rapeseed and other dicotyledonous plants, strong seed-specific promoters from a bean phaseolin gene, a soybean  $\beta$ -conglycinin gene, glycinin gene, Kunitz trypsin inhibitor gene, or rapeseed napin gene would be preferred. For corn and other monocotyledonous plants, a strong endosperm-specific promoter, e.g., the 10 kD or 27 kD zein promoter, would be preferred.

Transformed plants containing any of the chimeric LKR genes can be obtained by the methods described above. In order to obtain transformed plants that express a chimeric gene for cosuppression of LKR or antisense LKR, as well as a chimeric gene encoding lysine-insensitive DHDPS, the cosuppression or antisense LKR gene could be linked to the chimeric gene encoding lysine-insensitive DHDPS and the two genes could be introduced into plants via transformation. Alternatively, the chimeric gene for cosuppression of LKR or antisense LKR could be introduced into previously transformed plants that express lysine-insensitive DHDPS, or the cosuppression or antisense LKR gene could be introduced into normal plants and the transformants obtained could be crossed with plants that express lysine-insensitive DHDPS.

Design of Lysine-Rich Polypeptides

It may be desirable to convert the high levels of lysine produced into a form that is insensitive to breakdown, e.g., by incorporating it into a di-, tri- or oligopeptide, or a lysine-rich storage protein. No natural lysine-rich proteins are known.

One aspect of this invention is the design of polypeptides which can be expressed *in vivo* to serve as lysine-rich seed storage proteins. Polypeptides are linear polymers of amino acids where the  $\alpha$ -carboxyl group of one amino acid is covalently bound to the  $\alpha$ -amino group of the next amino acid in the chain. Non-covalent interactions among the residues in the chain and with the surrounding solvent determine the final conformation of the molecule. Those skilled in the art must consider electrostatic forces, hydrogen bonds, Van der Waals forces, hydrophobic interactions, and conformational preferences of individual amino acid residues in the design of a stable folded polypeptide chain [see for example: Creighton, (1984) *Proteins, Structures and Molecular Properties*, W. H. Freeman and Company, New York, pp. 133-197, or Schulz et al., (1979) *Principles of Protein Structure*, Springer Verlag, New York, pp. 27-45]. The number of interactions and their complexity suggest that the design process may be aided by the use of natural protein models where possible.

The synthetic storage proteins (SSPs) embodied in this invention are chosen to be polypeptides with the potential to be enriched in lysine relative to average levels of proteins in plant seeds. Lysine is a charged amino acid at physiological pH and is therefore found most often on the surface of protein molecules [Chotia, (1976) *Journal of Molecular Biology* 105:1-14]. To maximize lysine content, Applicants chose a molecular shape with a high surface-to-volume ratio for the

synthetic storage proteins embodied in this invention. The alternatives were either to stretch the common globular shape of most proteins to form a rod-like extended structure or to flatten the globular shape to a disk-like structure. Applicants chose the former configuration as there are several natural models for long rod-like proteins in the class of fibrous proteins [Creighton, (1984) Proteins, Structures and Molecular Properties, W.H. Freeman and Company, New York, p. 191].

Coiled-coils constitute a well-studied subset of the class of fibrous proteins [see Cohen et al., (1986) Trends Biochem. Sci. 11:245-248]. Natural examples are found in  $\alpha$ -keratins, paramyosin, light meromyosin and tropomyosin. These protein molecules consist of two parallel alpha helices twisted about each other in a left-handed supercoil. The repeat distance of this supercoil is 140 Å (compared to a repeat distance of 5.4 Å for one turn of the individual helices). The supercoil causes a slight skew ( $10^\circ$ ) between the axes of the two individual alpha helices.

In a coiled coil there are 3.5 residues per turn of the individual helices resulting in an exact 7 residue periodicity with respect to the superhelix axis (see Figure 1). Every seventh amino acid in the polypeptide chain therefore occupies an equivalent position with respect to the helix axis. Applicants refer to the seven positions in this heptad unit of the invention as (d e f g a b c) as shown in Figures 1 and 2a. This conforms to the conventions used in the coiled-coil literature.

The a and d amino acids of the heptad follow a 4,3 repeat pattern in the primary sequence and fall on one side of an individual alpha helix (See Figure 1). If the amino acids on one side of an alpha helix are all non-polar, that face of the helix is hydrophobic and

will associate with other hydrophobic surfaces as, for example, the non-polar face of another similar helix. A coiled-coil structure results when two helices dimerize such that their hydrophobic faces are aligned with each other (See Figure 2a).

The amino acids on the external faces of the component alpha helices (b, c, e, f, g) are usually polar in natural coiled-coils in accordance with the expected pattern of exposed and buried residue types in globular proteins [Schulz, et al., (1979) Principles of Protein Structure. Springer Verlag, New York, p. 12; Talbot, et al., (1982) Acc. Chem. Res. 15:224-230; Hodges et al., (1981) Journal of Biological Chemistry 256:1214-1224]. Charged amino acids are sometimes found forming salt bridges between positions e and g' or positions g and e' on the opposing chain (see Figure 2a).

Thus, two amphipathic helices like the one shown in Figure 1 are held together by a combination of hydrophobic interactions between the a, a', d, and d' residues and by salt bridges between e and g' and/or g and e' residues. The packing of the hydrophobic residues in the supercoil maintains the chains "in register". For short polypeptides comprising only a few turns of the component alpha helical chains, the 10° skew between the helix axes can be ignored and the two chains treated as parallel (as shown in Figure 2a).

A number of synthetic coiled-coils have been reported in the literature (Lau et al., (1984) Journal of Biological Chemistry 259:13253-13261; Hodges et al., (1988) Peptide Research 1:19-30; DeGrado et al., (1989) Science 243:622-628; O'Neil et al., (1990) Science 250:646-651]. Although these polypeptides vary in size, Lau et al. found that 29 amino acids were sufficient for dimerization to form the coiled-coil structure [Lau et

al., (1984) Journal of Biological Chemistry 259:13253-13261]. Applicants constructed the polypeptides in this invention as 28-residue and larger chains for reasons of conformational stability.

5       The polypeptides of this invention are designed to dimerize with a coiled-coil motif in aqueous environments. Applicants have used a combination of hydrophobic interactions and electrostatic interactions to stabilize the coiled-coil conformation. Most  
10 nonpolar residues are restricted to the a and d positions which creates a hydrophobic stripe parallel to the axis of the helix. This is the dimerization face. Applicants avoided large, bulky amino acids along this face to minimize steric interference with dimerization  
15 and to facilitate formation of the stable coiled-coil structure.

      Despite recent reports in the literature suggesting that methionine at positions a and d is destabilizing to coiled-coils in the leucine zipper subgroup [Landschulz  
20 et al., (1989) Science 243:1681-1688 and Hu et al., (1990) Science 250:1400-1403], Applicants chose to substitute methionine residues for leucine on the hydrophobic face of the SSP polypeptides. Methionine and leucine are similar in molecular shape (Figure 3).  
25 Applicants demonstrated that any destabilization of the coiled-coil that may be caused by methionine in the hydrophobic core appears to be compensated in sequences where the formation of salt bridges (e-g' and g-e') occurs at all possible positions in the helix (i.e.,  
30 twice per heptad).

      To the extent that it is compatible with the goal of creating a polypeptide enriched in lysine, Applicants minimized the unbalanced charges in the polypeptide. This may help to prevent undesirable interactions



between the synthetic storage proteins and other plant proteins when the polypeptides are expressed in vivo.

The polypeptides of this invention are designed to spontaneously fold into a defined, conformationally  
5 stable structure, the alpha helical coiled-coil, with minimal restrictions on the primary sequence. This allows synthetic storage proteins to be custom-tailored for specific end-user requirements. Any amino acid can be incorporated at a frequency of up to one in every  
10 seven residues using the b, c, and f positions in the heptad repeat unit. Applicants note that up to 43% of an essential amino acid from the group isoleucine, leucine, lysine, methionine, threonine, and valine can be incorporated and that up to 14% of the essential  
15 amino acids from the group phenylalanine, tryptophan, and tyrosine can be incorporated into the synthetic storage proteins of this invention.

In the SSPs only Met, Leu, Ile, Val or Thr are located in the hydrophobic core. Furthermore, the e, g,  
20 e', and g' positions in the SSPs are restricted such that an attractive electrostatic interaction always occurs at these positions between the two polypeptide chains in an SSP dimer. This makes the SSP polypeptides more stable as dimers.

25 Thus, the novel synthetic storage proteins described in this invention represent a particular subset of possible coiled-coil polypeptides. Not all polypeptides which adopt an amphipathic alpha helical conformation in aqueous solution are suitable for the  
30 applications described here.

The following rules derived from Applicants' work define the SSP polypeptides that Applicants use in their invention:

The synthetic polypeptide comprises n heptad units (d e f g a b c), each heptad being either the same or different, wherein:

n is at least 4;

5 a and d are independently selected from the group consisting of Met, Leu, Val, Ile and Thr;

e and g are independently selected from the group consisting of the acid/base pairs  
10 Glu/Lys, Lys/Glu, Arg/Glu, Arg/Asp, Lys/Asp, Glu/Arg, Asp//Arg and Asp/Lys; and

b, c and f are independently any amino acids except Gly or Pro and at least two amino acids of b, c and f in each heptad are  
15 selected from the group consisting of Glu, Lys, Asp, Arg, His, Thr, Ser, Asn, Gln, Cys and Ala.

#### Chimeric Genes Encoding Lysine-Rich Polypeptides

20 DNA sequences which encode the polypeptides described above can be designed based upon the genetic code. Where multiple codons exist for particular amino acids, codons should be chosen from those preferable for translation in plants. Oligonucleotides corresponding  
25 to these DNA sequences can be synthesized using an ABI DNA synthesizer, annealed with oligonucleotides corresponding to the complementary strand and inserted into a plasmid vector by methods known to those skilled in the art. The encoded polypeptide sequences can be  
30 lengthened by inserting additional annealed oligonucleotides at restriction endonuclease sites engineered into the synthetic gene. Some representative strategies for constructing genes encoding lysine-rich polypeptides of the invention, as well as DNA and amino acid sequences  
35 of preferred embodiments are provided in Example 8.

A chimeric gene designed to express RNA for a synthetic storage protein gene encoding a lysine-rich polypeptide can be constructed by linking the gene to any of the plant promoter sequences described above.

- 5 Preferred promoters would be seed-specific promoters. For soybean, rapeseed and other dicotyledonous plants strong seed-specific promoters from a bean phaseolin gene, a soybean  $\beta$ -conglycinin gene, glycinin gene, Kunitz trypsin inhibitor gene, or rapeseed napin gene  
10 would be preferred. For corn or other monocotyledonous plants, a strong endosperm-specific promoter, e.g., the 10 kD or 27 kD zein promoter, or a strong embryo-specific promoter, e.g., the corn globulin 1 promoter, would be preferred.

- 15 In order to obtain plants that express a chimeric gene for a synthetic storage protein gene encoding a lysine-rich polypeptide, plants can be transformed by any of the methods described above. In order to obtain plants that express both a chimeric SSP gene and a  
20 chimeric gene encoding lysine-insensitive DHDPS, the SSP gene could be linked to the chimeric gene encoding lysine-insensitive DHDPS and the two genes could be introduced into plants via transformation. Alternatively, the chimeric SSP gene could be introduced  
25 into previously transformed plants that express lysine-insensitive DHDPS, or the SSP gene could be introduced into normal plants and the transformants obtained could be crossed with plants that express lysine-insensitive DHDPS.

- 30 Results from genetic crosses of transformed plants containing lysine biosynthesis genes with transformed plants containing lysine-rich protein genes (see Example 10) demonstrate that the total lysine levels in seeds can be increased by the coordinate expression of  
35 these genes. This result was especially striking

because the gene copy number of all of the transgenes was reduced in the hybrid. It is expected that the lysine level would be further increased if the biosynthesis genes and the lysine-rich protein genes were all homozygous.

#### EXAMPLES

The present invention is further defined in the following Examples, in which all parts and percentages are by weight and degrees are Celsius, unless otherwise stated.

#### EXAMPLE 1

##### Isolation of the E. coli and Corynebacterium glutamicum dapA genes

The *E. coli* *dapA* gene (*ecodapA*) has been cloned, restriction endonuclease mapped and sequenced previously [Richaud et al. (1986) J. Bacteriol. 166:297-300]. For the present invention the *dapA* gene was obtained on a bacteriophage lambda clone from an ordered library of 3400 overlapping segments of cloned *E. coli* DNA constructed by Kohara, Akiyama and Isono [Kohara et al. (1987) Cell 50:595-508]. From the knowledge of the map position of *dapA* at 53 min on the *E. coli* genetic map [Bachman (1983) Microbiol. Rev. 47:180-230], the restriction endonuclease map of the cloned gene [Richaud et al. (1986) J. Bacteriol. 166:297-300], and the restriction endonuclease map of the cloned DNA fragments in the *E. coli* library [Kohara et al. (1987) Cell 50:595-508], it was possible to choose lambda phages 4C11 and 5A8 [Kohara et al. (1987) Cell 50:595-508] as likely candidates for carrying the *dapA* gene. The phages were grown in liquid culture from single plaques as described [see Current Protocols in Molecular Biology (1987) Ausubel et al. eds., John Wiley & Sons New York] using LE392 as host [see Sambrook et al. (1989) Molecular Cloning: a Laboratory Manual, Cold Spring

Harbor Laboratory Press]. Phage DNA was prepared by phenol extraction as described [see Current Protocols in Molecular Biology (1987) Ausubel et al. eds., John Wiley & Sons, New York]. Both phages contained an

5 approximately 2.8 kb Pst I DNA fragment expected for the dapA gene [Richaud et al. (1986) J. Bacteriol. 166:297-300]. The fragment was isolated from the digest of phage 5A8 and inserted into Pst I digested vector pBR322 yielding plasmid pBT427.

10 The Corynebacterium dapA gene (cordapA) was isolated from genomic DNA from ATCC strain 13032 using polymerase chain reaction (PCR). The nucleotide sequence of the Corynebacterium dapA gene has been published [Bonnassie et al. (1990) Nucleic Acids Res. 15 18:6421]. From the sequence it was possible to design oligonucleotide primers for PCR that would allow amplification of a DNA fragment containing the gene, and at the same time add unique restriction endonuclease sites at the start codon (Nco I) and just past the stop codon (EcoR I) of the gene. The oligonucleotide primers used were:

SEQ ID NO:1:

25 CCCGGGCCAT GGCTACAGGT TTAACAGCTA AGACCGGAGT AGAGCACT

SEQ ID NO:2:

GATATCGAAT TCTCATTATA GAACTCCAGC TTTTTC

30 PCR was performed using a Perkin-Elmer Cetus kit according to the instructions of the vendor on a thermocycler manufactured by the same company. The reaction product, when run on an agarose gel and stained with ethidium bromide, showed a strong DNA band of the size expected for the Corynebacterium dapA gene, about 35 900 bp. The PCR-generated fragment was digested with restriction endonucleases Nco I and EcoR I and inserted

into expression vector pBT430 (see Example 2) digested with the same enzymes. In addition to introducing an Nco I site at the translation start codon, the PCR primers also resulted in a change of the second codon from AGC coding for serine to GCT coding for alanine. Several clones that expressed active, lysine-insensitive DHDPS (see Example 2) were isolated, indicating that the second codon amino acid substitution did not affect activity; one clone was designated FS766.

10 The Nco I to EcoR I fragment carrying the PCR-generated Corynebacterium dapA gene was subcloned into the phagemid vector pGEM-9Zf(-) from Promega, single-stranded DNA was prepared and sequenced. This sequence is shown in SEQ ID NO:3.

15 Aside from the differences in the second codon already mentioned, the sequence matched the published sequence except at two positions, nucleotides 798 and 799. In the published sequence these are TC, while in the gene shown in SEQ ID NO:3 they are CT. This change results in an amino acid substitution of leucine for serine. The reason for this difference is not known. It may be due to an error in the published sequence, the difference in strains used to isolate the gene, or a PCR-generated error. The latter seems unlikely since 20 the same change was observed in at least 3 independently isolated PCR-generated dapA genes. The difference has no apparent effect on DHDPS enzyme activity (see Example 2).

#### EXAMPLE 2

30 High level expression of the E. coli and Corynebacterium glutamicum dapA genes in E. coli

An Nco I (CCATGG) site was inserted at the translation initiation codon of the E. coli dapA gene using oligonucleotide-directed mutagenesis. The 2.8 kb 35 Pst I DNA fragment carrying the dapA gene in plasmid

pBT427 (see Example 1) was inserted into the Pst I site of phagemid vector pTZ18R (Pharmacia) yielding pBT431. The orientation of the dapA gene was such that the coding strand would be present on the single-stranded  
5 phagemid DNA. Oligonucleotide-directed mutagenesis was carried out using a Muta-Gene kit from Bio-Rad according to the manufacturer's protocol with the mutagenic primer shown below:

10 SEQ ID NO:4:  
CTTCCCGTGA CCATGGGCCA TC

Putative mutants were screened for the presence of an Nco I site and a plasmid, designated pBT437, was shown  
15 to have the the proper sequence in the vicinity of the mutation by DNA sequencing. The addition of an Nco I site at the translation start codon also resulted in a change of the second codon from TTC coding for phenylalanine to GTC coding for valine.

20 To achieve high level expression of the dapA genes in E. coli the bacterial expression vector pBT430. This expression vector is a derivative of pET-3a [Rosenberg et al. (1987) Gene 56:125-135] which employs the bacteriophage T7 RNA polymerase/T7 promoter system.  
25 Plasmid pBT430 was constructed by first destroying the EcoR I and Hind III sites in pET-3a at their original positions. An oligonucleotide adaptor containing EcoR I and Hind III sites was inserted at the BamH I site of pET-3a. This created pET-3aM with additional unique  
30 cloning sites for insertion of genes into the expression vector. Then, the Nde I site at the position of translation initiation was converted to an Nco I site using oligonucleotide-directed mutagenesis. The DNA sequence of pET-3aM in this region, 5'-CATATGG, was  
35 converted to 5'-CCCATGG in pBT430.

The *E. coli* dapA gene was cut out of plasmid pBT437 as an 1150 bp Nco I-Hind III fragment and inserted into the expression vector pBT430 digested with the same enzymes, yielding plasmid pBT442. For expression of the  
5 *Corynebacterium* dapA gene, the 917 bp Nco I to EcoR I fragment of SEQ ID NO:3 inserted in pBT430 (pFS766, see Example 1) was used.

For high level expression each of the plasmids was transformed into *E. coli* strain BL21(DE3) [Studier  
10 et al. (1986) J. Mol. Biol. 189:113-130]. Cultures were grown in LB medium containing ampicillin (100 mg/L) at 25°C. At an optical density at 600 nm of approximately 1, IPTG (isopropylthio- $\beta$ -galactoside, the inducer) was added to a final concentration of 0.4 mM and incubation  
15 was continued for 3 h at 25°C. The cells were collected by centrifugation and resuspended in 1/20th (or 1/100th) the original culture volume in 50 mM NaCl; 50 mM Tris-Cl, pH 7.5; 1 mM EDTA, and frozen at -20°C. Frozen aliquots of 1 mL were thawed at 37°C and sonicated, in  
20 an ice-water bath, to lyse the cells. The lysate was centrifuged at 4°C for 5 min at 15,000 rpm. The supernatant was removed and the pellet was resuspended in 1 mL of the above buffer.

The supernatant and pellet fractions of uninduced  
25 and IPTG-induced cultures of BL21(DE3)/pBT442 or BL21(DE3)/pFS766 were analyzed by SDS polyacrylamide gel electrophoresis. The major protein visible by Coomassie blue staining in the supernatant and pellet fractions of both induced cultures had a molecular weight of  
30 32-34 kd, the expected size for DHDPS. Even in the uninduced cultures this protein was the most prominent protein produced.

In the BL21(DE3)/pBT442 IPTG-induced culture about  
80% of the DHDPS protein was in the supernatant and  
35 DHDPS represented 10-20% of the total protein in the



extract. In the BL21(DE3)/pFS766 IPTG-induced culture more than 50% of the DHDPS protein was in the pellet fraction. The pellet fractions in both cases were 90-95% pure DHDPS, with no other single protein present in significant amounts. Thus, these fractions were pure enough for use in the generation of antibodies. The pellet fractions containing 2-4 milligrams of either *E. coli* DHDPS or *Corynebacterium* DHDPS were solubilized in 50 mM NaCl; 50 mM Tris-Cl, pH 7.5; 1 mM EDTA, 0.2 mM dithiothreitol, 0.2% SDS and sent to Hazelton Research Facility (310 Swampridge Road, Denver, PA 17517) to have rabbit antibodies raised against the proteins.

DHDPS enzyme activity was assayed as follows:  
Assay mix (for 10 X 1.0 mL assay tubes or 40 X 0.25 mL for microtiter dish); made fresh, just before use:

2.5mL	H <sub>2</sub> O
0.5mL	1.0M Tris-HCl pH8.0
0.5mL	0.1M Na Pyruvate
0.5mL	o-Aminobenzaldehyde (10mg/mL in ethanol)
25μL	1.0M DL-Aspartic-β-semialdehyde (ASA) in 1.0N HCl

Assay (1.0mL): MicroAssay (0.25mL):

25	DHDPS assay mix	0.40mL	0.10mL
	enzyme extract + H <sub>2</sub> O;	0.10mL	.025mL
	10mM L-lysine	5μL or 20μL	1μL or 5μL

Incubate at 30°C for desired time. Stop by addition of:

30	1.0N HCl	0.50mL	0.125mL
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Color allowed to develop for 30-60 min. Precipitate spun down in eppendorf centrifuge. OD<sub>540</sub> vs 0 min read as blank. For MicroAssay, aliquot 0.2 mL into microtiter well and read at OD<sub>530</sub>.

The specific activity of *E. coli* DHDPS in the supernatant fraction of induced extracts was about 50 OD<sub>540</sub> units per minute per milligram protein in a 1.0 mL assay. *E. coli* DHDPS was sensitive to the presence of L-lysine in the assay. Fifty percent inhibition was found at a concentration of about 0.5 mM. For *Corynebacterium* DHDPS, the activity was measured in the supernatant fraction of uninduced extracts, rather than induced extracts. Enzyme activity was about 4 OD<sub>530</sub> units per minute per milligram protein in a 0.25 mL assay. In contrast to *E. coli* DHDPS, *Corynebacterium* DHDPS was not inhibited at all by L-lysine, even at a concentration of 70 mM.

#### EXAMPLE 3

##### Isolation of the *E. coli* lysC Gene and mutations in lysC resulting in lysine-insensitive AKIII

The *E. coli* *lysC* gene has been cloned, restriction endonuclease mapped and sequenced previously [Cassan et al. (1986) J. Biol. Chem. 261:1052-1057]. For the present invention the *lysC* gene was obtained on a bacteriophage lambda clone from an ordered library of 3400 overlapping segments of cloned *E. coli* DNA constructed by Kohara, Akiyama and Isono [Kohara et al. (1987) Cell 50:595-508]. This library provides a physical map of the whole *E. coli* chromosome and ties the physical map to the genetic map. From the knowledge of the map position of *lysC* at 90 min. on the *E. coli* genetic map [Theze et al. (1974) J. Bacteriol. 117:133-143], the restriction endonuclease map of the cloned gene [Cassan et al. (1986) J. Biol. Chem. 261:1052-1057], and the restriction endonuclease map of the cloned DNA fragments in the *E. coli* library [Kohara et al. (1987) Cell 50:595-508], it was possible to choose lambda phages 4E5 and 7A4 [Kohara et al. (1987) Cell 50:595-508] as likely candidates for carrying the

lysC gene. The phages were grown in liquid culture from single plaques as described [see Current Protocols in Molecular Biology (1987) Ausubel et al. eds. John Wiley & Sons New York] using LE392 as host [see Sambrook et al. (1989) Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press]. Phage DNA was prepared by phenol extraction as described [see Current Protocols in Molecular Biology (1987) Ausubel et al. eds. John Wiley & Sons, New York].

From the sequence of the gene several restriction endonuclease fragments diagnostic for the lysC gene were predicted, including an 1860 bp EcoR I-Nhe I fragment, a 2140 bp EcoR I-Xmn I fragment and a 1600 bp EcoR I-BamH I fragment. Each of these fragments was detected in both of the phage DNAs confirming that these carried the lysC gene. The EcoR I-Nhe I fragment was isolated and subcloned in plasmid pBR322 digested with the same enzymes, yielding an ampicillin-resistant, tetracycline-sensitive *E. coli* transformant. The plasmid was designated pBT436.

To establish that the cloned lysC gene was functional, pBT436 was transformed into *E. coli* strain Gif106M1 (*E. coli* Genetic Stock Center strain CGSC-5074) which has mutations in each of the three *E. coli* AK genes [Theze et al. (1974) J. Bacteriol. 117:133-143]. This strain lacks all AK activity and therefore requires diaminopimelate (a precursor to lysine which is also essential for cell wall biosynthesis), threonine and methionine. In the transformed strain all these nutritional requirements were relieved demonstrating that the cloned lysC gene encoded functional AKIII.

Addition of lysine (or diaminopimelate which is readily converted to lysine in vivo) at a concentration of approximately 0.2 mM to the growth medium inhibits the growth of Gif106M1 transformed with pBT436. M9

media [see Sambrook et al. (1989) Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press] supplemented with the arginine and isoleucine, required for Gif106M1 growth, and ampicillin, to maintain selection for the pBT436 plasmid, was used. This inhibition is reversed by addition of threonine plus methionine to the growth media. These results indicated that AKIII could be inhibited by exogenously added lysine leading to starvation for the other amino acids derived from aspartate. This property of pBT436-transformed Gif106M1 was used to select for mutations in lysC that encoded lysine-insensitive AKIII.

Single colonies of Gif106M1 transformed with pBT436 were picked and resuspended in 200  $\mu$ L of a mixture of 100  $\mu$ L 1% lysine plus 100  $\mu$ L of M9 media. The entire cell suspension containing  $10^7$ - $10^8$  cells was spread on a petri dish containing M9 media supplemented with the arginine, isoleucine, and ampicillin. Sixteen petri dishes were thus prepared. From 1 to 20 colonies appeared on 11 of the 16 petri dishes. One or two (if available) colonies were picked and retested for lysine resistance and from this nine lysine-resistant clones were obtained. Plasmid DNA was prepared from eight of these and re-transformed into Gif106M1 to determine whether the lysine resistance determinant was plasmid-borne. Six of the eight plasmid DNAs yielded lysine-resistant colonies. Three of these six carried lysC genes encoding AKIII that was uninhibited by 15mM lysine, whereas wild type AKIII is 50% inhibited by 0.3-0.4 mM lysine and >90% inhibited by 1 mM lysine (see Example 2 for details).

To determine the molecular basis for lysine-resistance the sequences of the wild type lysC gene and three mutant genes were determined. A method for "Using mini-prep plasmid DNA for sequencing double stranded

templates with sequenase™ [Kraft et al. (1988) BioTechniques 6:544-545] was used. Oligonucleotide primers, based on the published lysC sequence and spaced approximately every 200 bp, were synthesized to facilitate the sequencing. The sequence of the wild type lysC gene cloned in pBT436 (SEQ ID NO:5) differed from the published lysC sequence in the coding region at 5 positions. Four of these nucleotide differences were at the third position in a codon and would not result in a change in the amino acid sequence of the AKIII protein. One of the differences would result in a cysteine to glycine substitution at amino acid 58 of AKIII. These differences are probably due to the different strains from which the lysC genes were cloned.

The sequences of the three mutant lysC genes that encoded lysine-insensitive AK each differed from the wild type sequence by a single nucleotide, resulting in a single amino acid substitution in the protein. Mutant M2 had an A substituted for a G at nucleotide 954 of SEQ ID NO:5 resulting in an isoleucine for methionine substitution at amino acid 318 and mutants M3 and M4 had identical T for C substitutions at nucleotide 1055 of SEQ ID NO:5 resulting in an isoleucine for threonine substitution at amino acid 352. Thus, either of these single amino acid substitutions is sufficient to render the AKIII enzyme insensitive to lysine inhibition.

An Nco I (CCATGG) site was inserted at the translation initiation codon of the lysC gene using the following oligonucleotides:

SEQ ID NO:6:  
GATCCATGGC TGAAATTGTT GTCTCCAAAT TTGGCG

SEQ ID NO:7:  
GTACCGCCAA ATTTGGAGAC AACAAATTCA GCCATG

When annealed these oligonucleotides have BamH I and Asp 718 "sticky" ends. The plasmid pBT436 was digested with BamH I, which cuts upstream of the lysC coding sequence and Asp 718 which cuts 31 nucleotides downstream of the initiation codon. The annealed oligonucleotides were ligated to the plasmid vector and E. coli transformants were obtained. Plasmid DNA was prepared and screened for insertion of the oligonucleotides based on the presence of an Nco I site. A plasmid containing the site was sequenced to assure that the insertion was correct, and was designated pBT457. In addition to creating an Nco I site at the initiation codon of lysC, this oligonucleotide insertion changed the second codon from TCT, coding for serine, to GCT, coding for alanine. This amino acid substitution has no apparent effect on the AKIII enzyme activity.

The lysC gene was cut out of plasmid pBT457 as a 1560 bp Nco I-EcoR I fragment and inserted into the expression vector pBT430 digested with the same enzymes, yielding plasmid pBT461. For expression of the mutant lysC-M4 gene pBT461 was digested with Kpn I-EcoR I, which removes the wild type lysC gene from about 30 nucleotides downstream from the translation start codon, and inserting the analogous Kpn I-EcoR I fragments from the mutant genes yielding plasmid pBT492.

#### EXAMPLE 4

##### Construction of Chimeric dapA

##### Genes for Expression in the Seeds of Plants

A seed-specific expression cassette (Figure 4) is composed of the promoter and transcription terminator from the gene encoding the  $\beta$  subunit of the seed storage protein phaseolin from the bean Phaseolus vulgaris [Doyle et al. (1986) J. Biol. Chem. 261:9228-9238]. The phaseolin cassette includes about 500 nucleotides upstream (5') from the translation initiation codon and

about 1650 nucleotides downstream (3') from the translation stop codon of phaseolin. Between the 5' and 3' regions are the unique restriction endonuclease sites Nco I (which includes the ATG translation initiation codon), Sma I, Kpn I and Xba I. The entire cassette is flanked by Hind III sites.

Plant amino acid biosynthetic enzymes are known to be localized in the chloroplasts and therefore are synthesized with a chloroplast targeting signal. Bacterial proteins such as DHDPS and AKIII have no such signal. A chloroplast transit sequence (cts) was therefore fused to the dapA and lysC-M4 coding sequence in the chimeric genes. The cts used was based on the the cts of the small subunit of ribulose 1,5-bisphosphate carboxylase from soybean [Berry-Lowe et al. (1982) J. Mol. Appl. Genet. 1:483-498]. The oligonucleotides SEQ ID NOS:8-11 were synthesized and used as described below.

Three chimeric genes were created:

- No. 1) phaseolin 5' region/cts/lysC-M4/phaseolin 3' region
- No. 2) phaseolin 5' region/cts/ecodapA/phaseolin 3' region
- No. 3) phaseolin 5' region/cts/cordapA/phaseolin 3' region

Oligonucleotides SEQ ID NO:8 and SEQ ID NO:9, which encode the carboxy terminal part of the chloroplast targeting signal, were annealed, resulting in Nco I compatible ends, purified via polyacrylamide gel electrophoresis, and inserted into Nco I digested pBT461. The insertion of the correct sequence in the correct orientation was verified by DNA sequencing yielding pBT496. Oligonucleotides SEQ ID NO:10 and SEQ ID NO:11, which encode the amino terminal part of the chloroplast targeting signal, were annealed, resulting

in Nco I compatible ends, purified via polyacrylamide gel electrophoresis, and inserted into Nco I digested pBT496. The insertion of the correct sequence in the correct orientation was verified by DNA sequencing  
5 yielding pBT521. Thus the cts was fused to the lysC gene.

To fuse the cts to the lysC-M4 gene, pBT521 was digested with Sal I, and an approximately 900 bp DNA fragment that included the cts and the amino terminal  
10 coding region of lysC was isolated. This fragment was inserted into Sal I digested pBT492, effectively replacing the amino terminal coding region of lysC-M4 with the fused cts and the amino terminal coding region of lysC. Since the mutation that resulted in lysine-  
15 insensitivity was not in the replaced fragment, the new plasmid, pBT523, carried the cts fused to lysC-M4.

The 1600 bp Nco I-Hpa I fragment containing the cts fused to lysC-M4 plus about 90 bp of 3' non-coding sequence was isolated and inserted into the seed-  
20 specific expression cassette digested with Nco I and Sma I (chimeric gene No. 1), yielding plasmid pBT544.

Before insertion into the expression cassette, the ecodapA gene was modified to insert a restriction endonuclease site, Kpn I, just after the translation  
25 stop codon. The oligonucleotides SEQ ID NOS:12-13 were synthesized for this purpose:

SEQ ID NO:12:

CCGGTTTGCT GTAATAGGTA CCA  
30

SEQ ID NO:13:

AGCTTGGTAC CTATTACAGC AAACCGGCAT G

Oligonucleotides SEQ ID NO:12 and SEQ ID NO:13 were  
35 annealed, resulting in an Sph I compatible end on one end and a Hind III compatible end on the other and



inserted into Sph I plus Hind III digested pBT437. The insertion of the correct sequence was verified by DNA sequencing yielding pBT443.

5 An 880 bp Nco I-Kpn I fragment from pBT443 containing the entire ecodapA coding region was isolated from an agarose gel following electrophoresis and inserted into the seed-specific expression cassette digested with Nco I and Kpn I, yielding plasmid pBT494. Oligonucleotides SEQ ID NO:8-11 were used as described  
10 above to add a cts to the ecodapA coding region in the seed-specific expression cassette, yielding chimeric gene No. 2 in pBT520.

An 870 bp Nco I-EcoR I fragment from pFS766 containing the entire cordapA coding region was isolated  
15 from an agarose gel following electrophoresis and inserted into the leaf expression cassette digested with Nco I and EcoR I, yielding plasmid pFS789. To attach the cts to the cordapA gene a DNA fragment containing the entire cts was prepared using PCR. The template DNA  
20 was pBT544 and the oligonucleotide primers used were:

SEQ ID NO:14:  
GCTTCCTCAA TGATCTCCTC CCCAGCT

25 SEQ ID NO:15:  
CATTGTACTC TTCCACCGTT GCTAGCAA

PCR was performed using a Perkin-Elmer Cetus kit according to the instructions of the vendor on a  
30 thermocycler manufactured by the same company. The PCR-generated 160 bp fragment was treated with T4 DNA polymerase in the presence of the 4 deoxyribonucleotide triphosphates to obtain a blunt-ended fragment. The cts fragment was inserted into the Nco I containing the  
35 start codon of the cordapA gene which had been digested and treated with the Klenow fragment of DNA polymerase

to fill in the 5' overhangs. The inserted fragment and the vector/insert junctions were determined to be correct by DNA sequencing.

5 A 1030 bp Nco I-Kpn I fragment containing the cts attached to the cordapA coding region was isolated from an agarose gel following electrophoresis and inserted into the phaseolin seed expression cassette digested with Nco I and Kpn I, yielding plasmid pFS889 containing chimeric gene No. 3.

10

#### EXAMPLE 5

##### Transformation of Rapeseed with the Phaseolin Promoter/cts/cordapA and

##### Phaseolin Promoter/cts/lysC-M4 Chimeric Genes

15 The chimeric gene cassettes, phaseolin 5' region/ cts/cordapA/phaseolin 3' region, phaseolin 5' region/ cts/lysC-M4/phaseolin 3', and phaseolin 5' region/ cts/cordapA/phaseolin 3' region plus phaseolin 5' region/cts/lysC-M4/phaseolin 3' (Example 4) were inserted into the binary vector pZS199 (Figure 5A). In  
20 pZS199 the 35S promoter from Cauliflower Mosaic Virus drives expression of the NPT II.

The phaseolin 5' region/cts/cordapA/phaseolin 3' region chimeric gene cassette was modified using oligonucleotide adaptors to convert the Hind III sites  
25 at each end to BamH I sites. The gene cassette was then isolated as a 2.7 kb BamH I fragment and inserted into BamH I digested pZS199, yielding plasmid pFS926 (Figure 5B). This binary vector has the chimeric gene, phaseolin 5' region/cts/cordapA/phaseolin 3' region  
30 inserted in the same orientation as the 35S/NPT II/nos 3' marker gene.

To insert the phaseolin 5' region/cts/lysC-M4/phaseolin 3' region, the gene cassette was isolated as a 3.3 kb EcoR I to Spe I fragment and inserted into  
35 EcoR I plus Xba I digested pZS199, yielding plasmid

pBT593. This binary vector has the chimeric gene, phaseolin 5' region/cts/lysC-M4/phaseolin 3' region inserted in the same orientation as the 35S/NPT II/nos 3' marker gene.

5 To combine the two cassettes, the EcoR I site of pBT593 was converted to a BamH I site using oligonucleotide adaptors, the resulting vector was cut with BamH I and the phaseolin 5' region/cts/cordapA/phaseolin 3' region gene cassette was isolated as a  
10 2.7 kb BamH I fragment and inserted, yielding pBT597. This binary vector has both chimeric genes, phaseolin 5' region/cts/cordapA/phaseolin 3' region and phaseolin 5' region/cts/lysC-M4/phaseolin 3' region inserted in the same orientation as the 35S/NPT II/nos 3' marker gene.

15 Brassica napus cultivar "Westar" was transformed by co-cultivation of seedling pieces with disarmed Agrobacterium tumefaciens strain LBA4404 carrying the the appropriate binary vector.

B. napus seeds were sterilized by stirring in 10%  
20 Chlorox, 0.1% SDS for thirty min, and then rinsed thoroughly with sterile distilled water. The seeds were germinated on sterile medium containing 30 mM CaCl<sub>2</sub> and 1.5% agar, and grown for six d in the dark at 24°C.

Liquid cultures of Agrobacterium for plant  
25 transformation were grown overnight at 28°C in Minimal A medium containing 100 mg/L kanamycin. The bacterial cells were pelleted by centrifugation and resuspended at a concentration of 10<sup>8</sup> cells/mL in liquid Murashige and Skoog Minimal Organic medium containing 100 uM  
30 acetosyringone.

B. napus seedling hypocotyls were cut into 5 mm segments which were immediately placed into the bacterial suspension. After 30 min, the hypocotyl pieces were removed from the bacterial suspension and  
35 placed onto BC-35 callus medium containing 100 uM

acetosyringone. The plant tissue and Agrobacteria were co-cultivated for three d at 24°C in dim light.

The co-cultivation was terminated by transferring the hypocotyl pieces to BC-35 callus medium containing  
5 200 mg/L carbenicillin to kill the Agrobacteria, and 25 mg/L kanamycin to select for transformed plant cell growth. The seedling pieces were incubated on this medium for three weeks at 24°C under continuous light.

After three weeks, the segments were transferred to  
10 BS-48 regeneration medium containing 200 mg/L carbenicillin and 25 mg/L kanamycin. Plant tissue was subcultured every two weeks onto fresh selective regeneration medium, under the same culture conditions described for the callus medium. Putatively transformed  
15 calli grew rapidly on regeneration medium; as calli reached a diameter of about 2 mm, they were removed from the hypocotyl pieces and placed on the same medium lacking kanamycin

Shoots began to appear within several weeks after  
20 transfer to BS-48 regeneration medium. As soon as the shoots formed discernable stems, they were excised from the calli, transferred to MSV-1A elongation medium, and moved to a 16:8-h photoperiod at 24°C.

Once shoots had elongated several internodes, they  
25 were cut above the agar surface and the cut ends were dipped in Rootone. Treated shoots were planted directly into wet Metro-Mix 350 soilless potting medium. The pots were covered with plastic bags which were removed when the plants were clearly growing, after about ten d.

30 Results of the transformation are shown in Table 1. Transformed plants were obtained with each of the binary vectors.

Minimal A Bacterial Growth Medium

Dissolve in distilled water:

- 10.5 g potassium phosphate, dibasic
- 4.5 g potassium phosphate, monobasic
- 5 1.0 g ammonium sulfate
- 0.5 g sodium citrate, dihydrate

Make up to 979 mL with distilled water

Autoclave

Add 20 mL filter-sterilized 10% sucrose

- 10 Add 1 mL filter-sterilized 1 M  $\text{MgSO}_4$

Brassica Callus Medium BC-35

Per liter:

- Murashige and Skoog Minimal Organic Medium
- 15 (MS salts, 100 mg/L i-inositol, 0.4 mg/L thiamine; GIBCO #510-3118)

- 30 g sucrose
- 18 g mannitol
- 0.5 mg/L 2,4-D
- 20 0.3 mg/L kinetin
- 0.6% agarose
- pH 5.8

Brassica Regeneration Medium BS-48

- 25 Murashige and Skoog Minimal Organic Medium
- Gamborg B5 Vitamins (SIGMA #1019)
- 10 g glucose
- 250 mg xylose
- 600 mg MES
- 30 0.4% agarose
- pH 5.7

Filter-sterilize and add after autoclaving:

- 2.0 mg/L zeatin
- 0.1 mg/L IAA
- 35

Brassica Shoot Elongation Medium MSV-1A

Murashige and Skoog Minimal Organic Medium

Gamborg B5 Vitamins

10 g sucrose

5 0.6% agarose

pH 5.8

TABLE 1  
Canola transformants

BINARY VECTOR	NUMBER OF CUT ENDS	NUMBER OF KAN <sup>R</sup> CALLI	NUMBER OF SHOOTING CALLI	NUMBER OF PLANTS
pZS199	120	41	5	2
pFS926	600	278	52	28
pBT593	600	70	10	3
pBT597	600	223	40	23

Plants were grown under a 16:8-h photoperiod, with  
 10 a daytime temperature of 23°C and a nighttime temperature  
 of 17°C. When the primary flowering stem began to  
 elongate, it was covered with a mesh pollen-containment  
 bag to prevent outcrossing. Self-pollination was  
 facilitated by shaking the plants several times each  
 15 day. Mature seeds derived from self-pollinations were  
 harvested about three months after planting.

A partially defatted seed meal was prepared as  
 follows: 40 milligrams of mature dry seed was ground  
 with a mortar and pestle under liquid nitrogen to a fine  
 20 powder. One milliliter of hexane was added and the  
 mixture was shaken at room temperature for 15 min. The  
 meal was pelleted in an eppendorf centrifuge, the hexane  
 was removed and the hexane extraction was repeated.  
 Then the meal was dried at 65° for 10 min until the  
 25 hexane was completely evaporated leaving a dry powder.  
 Total proteins were extracted from mature seeds as  
 follows. Approximately 30-40 mg of seeds were put into  
 a 1.5 mL disposable plastic microfuge tube and ground in

0.25 mL of 50 mM Tris-HCl pH 6.8, 2 mM EDTA, 1% SDS, 1%  $\beta$ -mercaptoethanol. The grinding was done using a motorized grinder with disposable plastic shafts designed to fit into the microfuge tube. The resultant suspensions were centrifuged for 5 min at room temperature in a microfuge to remove particulates. Three volumes of extract was mixed with 1 volume of 4 X SDS-gel sample buffer (0.17M Tris-HCl pH6.8, 6.7% SDS, 16.7%  $\beta$ -mercaptoethanol, 33% glycerol) and 5  $\mu$ L from each extract were run per lane on an SDS polyacrylamide gel, with bacterially produced DHDPS or AKIII serving as a size standard and protein extracted from untransformed tobacco seeds serving as a negative control. The proteins were then electrophoretically blotted onto a nitrocellulose membrane. The membranes were exposed to the DHDPS or AKIII antibodies at a 1:5000 dilution of the rabbit serum using standard protocol provided by BioRad with their Immun-Blot Kit. Following rinsing to remove unbound primary antibody the membranes were exposed to the secondary antibody, donkey anti-rabbit Ig conjugated to horseradish peroxidase (Amersham) at a 1:3000 dilution. Following rinsing to remove unbound secondary antibody, the membranes were exposed to Amersham chemiluminescence reagent and X-ray film.

Eight of eight FS926 transformants and seven of seven BT597 transformants expressed the DHDPS protein. The single BT593 transformant and five of seven BT597 transformants expressed the AKIII-M4 protein (Table 2).

To measure free amino acid composition of the seeds, free amino acids were extracted from 40 milligrams of the defatted meal in 0.6 mL of methanol/chloroform/water mixed in ratio of 12v/5v/3v (MCW) at room temperature. The mixture was vortexed and then centrifuged in an eppendorf microcentrifuge for about 3 min. Approximately 0.6 mL of supernatant was

decanted and an additional 0.2 mL of MCW was added to the pellet which was then vortexed and centrifuged as above. The second supernatant, about 0.2 mL, was added to the first. To this, 0.2 mL of chloroform was added followed by 0.3 mL of water. The mixture was vortexed and then centrifuged in an eppendorf microcentrifuge for about 3 min, the upper aqueous phase, approximately 1.0 mL, was removed, and was dried down in a Savant Speed Vac Concentrator. The samples were hydrolyzed in 6N hydrochloric acid, 0.4%  $\beta$ -mercaptoethanol under nitrogen for 24 h at 110-120°C; 1/4 of the sample was run on a Beckman Model 6300 amino acid analyzer using post-column ninhydrin detection. Relative free amino acid levels in the seeds were compared as ratios of lysine or threonine to leucine, thus using leucine as an internal standard.

There was a good correlation between transformants expressing higher levels of DHDPS protein and those having higher levels of free lysine. The highest expressing lines showed a greater than 100-fold increase in free lysine level in the seeds. There has been no greater accumulation of free lysine due to expression of AKIII-M4 along with Corynebacteria DHDPS compared to expression of Corynebacteria DHDPS alone. The transformant that expressed AKIII-M4 in the absence of Corynebacteria DHDPS showed a 5-fold increase in the level of free threonine in the seeds. A high level of  $\alpha$ -aminoadipic acid, indicative of lysine catabolism, was observed in many of the transformed lines. Thus, prevention of lysine catabolism by inactivation of lysine ketoglutarate reductase should further increase the accumulation of free lysine in the seeds. Alternatively, incorporation of lysine into a peptide or lysine-rich protein would prevent catabolism and lead to an increase in the accumulation of lysine in the seeds.



To measure the total amino acid composition of mature seeds, 2 milligrams of the defatted meal were hydrolyzed in 6N hydrochloric acid, 0.4%  $\beta$ -mercapto-ethanol under nitrogen for 24 h at 110-120°C; 1/100 of the sample was run on a Beckman Model 6300 amino acid analyzer using post-column ninhydrin detection. Relative amino acid levels in the seeds were compared as percentages of lysine, threonine or  $\alpha$ -aminoadipic acid to total amino acids. There was a good correlation between transformants expressing DHDPS protein and those having high levels of lysine. Seeds with a 5-100% increase in the lysine level, compared to the untransformed control, were observed. In the seeds with the highest levels, lysine makes up 11-13% of the total seed amino acids, considerably higher than any previously known rapeseed seed.

TABLE 2

FS926 Transformants: phaseolin 5' region/cts/cordapa/phaseolin 3'  
 BT593 Transformants: phaseolin 5' region/cts/lysC-M4/phaseolin 3'  
 BT597 Transformants: phaseolin 5' region/cts/lysC-M4/phaseolin 3'  
 phaseolin 5' region/cts/cordapa/phaseolin 3'

LINE	FREE AMINO ACIDS			WESTERN	WESTERN	% TOTAL AMINO		
	K/L	T/L	AA/L	CORYNE DHDPS	E. COLI AKIII-M4	K	T	AA
WESTAR	0.8	2.0	0	-	-	6.5	5.6	0
ZS199	1.3	3.2	0	-	-	6.3	5.4	0
FS926-3	140	2.0	16	++++	-	12	5.1	1.0
FS926-9	110	1.7	12	++++	-	11	5.0	0.8
FS926-11	7.9	2.0	5.2	++	-	7.7	5.2	0
FS926-6	14	1.8	4.6	+++	-	8.2	5.9	0
FS926-22	3.1	1.3	0.3	+	-	6.9	5.7	0
FS926-27	4.2	1.9	1.1	++	-	7.1	5.6	0
FS926-29	38	1.8	4.7	++++	-	12	5.2	1.6
FS926-68	4.2	1.8	0.9	++	-	8.3	5.5	0
BT593-42	1.4	11	0	-	++	6.3	6.0	0
BT597-14	6.0	2.6	4.3	++	+/-	7.0	5.3	0

BT597-145	1.3	2.9	0	+	-			
BT597-4	38	3.7	4.5	++++	++++	13	5.6	1.6
BT597-68	4.7	2.7	1.5	++	+	6.9	5.8	0
BT597-100	9.1	1.9	1.7	+++	++	6.6	5.7	0
BT597-148	7.6	2.3	0.9	+++	+	7.3	5.7	0
BT597-169	5.6	2.6	1.7	+++	+++	6.6	5.7	0

AA is  $\alpha$ -amino adipic acid

#### EXAMPLE 6

##### Transformation of Soybean with the Phaseolin Promoter/cts/cordapA and

##### Phaseolin Promoter/cts/lysC-M4 Chimeric Genes

The chimeric gene cassettes, phaseolin 5' region/cts/cordapA/phaseolin 3' region plus phaseolin 5' region/cts/lysC-M4/phaseolin 3', (Example 4) were inserted into the soybean transformation vector pBT603 (Figure 6A). This vector has a soybean transformation marker gene consisting of the 35S promoter from Cauliflower Mosaic Virus driving expression of the *E. coli*  $\beta$ -glucuronidase (GUS) gene [Jefferson et al. (1986) Proc. Natl. Acad. Sci. USA 83:8447-8451] with the Nos 3' region in a modified pGEM9Z plasmid.

To insert the phaseolin 5' region/cts/lysC-M4/phaseolin 3' region, the gene cassette was isolated as a 3.3 kb Hind III fragment and inserted into Hind III digested pBT603, yielding plasmid pBT609. This vector has the chimeric gene, phaseolin 5' region/cts/lysC-M4/phaseolin 3' region inserted in the opposite orientation from the 35S/GUS/Nos 3' marker gene.

The phaseolin 5' region/cts/cordapA/phaseolin 3' region chimeric gene cassette was modified using oligonucleotide adaptors to convert the Hind III sites at each end to BamH I sites. The gene cassette was then isolated as a 2.7 kb BamH I fragment and inserted into BamH I digested pBT609, yielding plasmid pBT614

(Figure 6B). This vector has both chimeric genes, phaseolin 5' region/cts/cordapA/phaseolin 3' region plus phaseolin 5' region/cts/lysC-M4/phaseolin 3' inserted in the same orientation, and both are in the opposite orientation from the 35S/GUS/Nos 3' marker gene.

Plasmid pBT614 was introduced into soybean via transformation by Agracetus Company (Middleton, WI), according to the procedure described in United States Patent No. 5,015,580. Seeds from five transformed lines were obtained and analyzed.

It was expected that the transgenes would be segregating in the R1 seeds of the transformed plants. To identify seeds that carried the transformation marker gene, a small chip of the seed was cut off with a razor and put into a well in a disposable plastic microtiter plate. A GUS assay mix consisting of 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM EDTA, 0.5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 0.1% Triton X-100, 0.5 mg/mL 5-Bromo-4-chloro-3-indolyl  $\beta$ -D-glucuronic acid was prepared and 0.15 mL was added to each microtiter well. The microtiter plate was incubated at 37° for 45 minutes. The development of blue color indicated the expression of GUS in the seed.

Four of five transformed lines showed approximately 3:1 segregation for GUS expression (Table 3). This indicates that the GUS gene was inserted at a single site in the soybean genome. The other transformant showed 9:1 segregation, suggesting that the GUS gene was inserted at two sites.

A meal was prepared from a fragment of individual seeds by grinding into a fine powder. Total proteins were extracted from the meal by adding 1 mg to 0.1 mL of 43 mM Tris-HCl pH 6.8, 1.7% SDS, 4.2%  $\beta$ -mercaptoethanol, 8% glycerol, vortexing the suspension, boiling for 2-3 minutes and vortexing again. The resultant suspensions were centrifuged for 5 min at room temperature in a

microfuge to remove particulates and 10  $\mu$ L from each extract were run per lane on an SDS polyacrylamide gel, with bacterially produced DHDPS or AKIII serving as a size standard. The proteins were then electro-  
5 phoretically blotted onto a nitrocellulose membrane. The membranes were exposed to the DHDPS or AKIII antibodies, at a 1:5000 or 1:1000 dilution, respectively, of the rabbit serum using standard protocol provided by BioRad with their Immun-Blot Kit.  
10 Following rinsing to remove unbound primary antibody the membranes were exposed to the secondary antibody, donkey anti-rabbit Ig conjugated to horseradish peroxidase (Amersham) at a 1:3000 dilution. Following rinsing to remove unbound secondary antibody, the membranes were  
15 exposed to Amersham chemiluminescence reagent and X-ray film.

Four of five transformants expressed the DHDPS protein. In the four transformants that expressed DHDPS, there was excellent correlation between  
20 expression of GUS and DHDPS in individual seeds (Table 3). Therefore, the GUS and DHDPS genes are integrated at the same site in the soybean genome. Two of five transformants expressed the AKIII protein, and again there was excellent correlation between expression  
25 of AKIII, GUS and DHDPS in individual seeds (Table 3). Thus, in these two transformants the GUS, AKIII and DHDPS genes are integrated at the same site in the soybean genome. One transformant expressed only GUS in its seeds.

30 To measure free amino acid composition of the seeds, free amino acids were extracted from 8-10 milligrams of the meal in 1.0 mL of methanol/chloro-  
form/water mixed in ratio of 12v/5v/3v (MCW) at room temperature. The mixture was vortexed and then  
35 centrifuged in an eppendorf microcentrifuge for about

3 min; approximately 0.8 mL of supernatant was decanted. To this supernatant, 0.2 mL of chloroform was added followed by 0.3 mL of water. The mixture was vortexed and then centrifuged in an eppendorf microcentrifuge for  
5 about 3 min, the upper aqueous phase, approximately 1.0 mL, was removed, and was dried down in a Savant Speed Vac Concentrator. The samples were hydrolyzed in 6N hydrochloric acid, 0.4%  $\beta$ -mercaptoethanol under  
10 nitrogen for 24 h at 110-120°C; 1/10 of the sample was run on a Beckman Model 6300 amino acid analyzer using post-column ninhydrin detection. Relative free amino acid levels in the seeds were compared as ratios of lysine to leucine, thus using leucine as an internal standard.

15        There was excellent correlation between transformants expressing Corynebacteria DHDPs protein and those having higher levels of free lysine. From 20 fold to 120-fold increases in free lysine level was observed in seeds expressing Corynebacteria DHDPs. A  
20 high level of saccharopine, indicative of lysine catabolism, was observed in seeds the contained high levels of lysine.

To measure the total amino acid composition of mature seeds, 1-1.4 milligrams of the seed meal was  
25 hydrolyzed in 6N hydrochloric acid, 0.4%  $\beta$ -mercaptoethanol under nitrogen for 24 h at 110-120°C; 1/50 of the sample was run on a Beckman Model 6300 amino acid analyzer using post-column ninhydrin detection. Lysine (and other amino acid) levels in the seeds were compared  
30 as percentages of the total amino acids.

There was excellent correlation between seeds expressing Corynebacteria DHDPs protein and those having high levels of lysine. Seeds with a 5-35% increase in the lysine level, compared to the untransformed control,  
35 were observed. In these seeds lysine makes up 7.5-7.7%

of the total seed amino acids, considerably higher than any previously known soybean seed.

TABLE 3

LINE-SEED	GUS	Free LYS/LEU	DWDPS	AKIII	% LYS TOT
A2396-145-4	-	0.9	-	-	5.75
A2396-145-8	-	1	-	-	
A2396-145-5	-	0.8			5.85
A2396-145-3	-	1			
A2396-145-9	+	2			
A2396-145-6	+	4.6			
A2396-145-1	+	8.7			
A2396-145-10	+	18.4			7.54
A2396-145-7	+	21.7	+	-	6.68
A2396-145-2	+	45.5	+	-	7.19
A5403-175-9	-	1.3			
A5403-175-4	-	1.2			6.01
A5403-175-3	-	1			6.02
A5403-175-7	+	1.5			
A5403-175-5	+	1.8			
A5403-175-1	+	6.2			
A5403-175-2	+	6.5			6.3
A5403-175-6	+	14.4			
A5403-175-8	+	47.8	+	-	7.67
A5403-175-10	+	124.3	+	-	7.49
A5403-181-9	+	1.4			
A5403-181-10	+	1.4			5.68
A5403-181-8	+	0.9			
A5403-181-6	+	1.5			
A5403-181-4	-	0.7	-	-	5.85
A5403-181-5	+	1.1			
A5403-181-2	-	1.8	-	-	5.59
A5403-181-3	+	2.7	-	-	5.5

65

A5403-181-7	+	1.9			
A5403-181-1	-	2.3			
A5403-183-9	-	0.8			
A5403-183-6	-	0.7	-	-	6.03
A5403-183-8	-	1.3			
A5403-183-4	-	1.3	-	-	6.04
A5403-183-5	+	0.9			
A5403-183-3	+	3.1			
A5403-183-1	+	3.3			
A5403-183-7	+	9.9			
A5403-183-10	+	22.3	+	+	6.74
A5403-183-2	+	23.1	+	+	7.3
A5403-196-8	-	0.9	-	-	5.92
A5403-196-6	+	8.3			
A5403-196-1	+	16.1	+	+	6.83
A5403-196-7	+	27.9			
A5403-196-3	+	52.8			
A5403-196-5	+	26			
A5403-196-2	+	16.2	+	+	
A5403-196-10	+	29	+	+	7.53
A5403-196-4	+	58.2	+	+	7.57
A5403-196-9	+	47.1			
wild type control	-	0.9	-	-	5.63

Eighteen additional transformed soybean lines were obtained. Single seeds from the lines were analyzed for GUS activity as described above, and all lines exhibited GUS-positive seeds. Meal was prepared from single seeds, or in some cases a pool of several seeds, and assayed for expression of DHDPS and AKIII proteins via western blot. Seventeen of the eighteen lines expressed DHDPS, and fifteen of the eighteen expressed AKIII.

Again there was excellent correlation between seeds

expressing GUS, DHDPS and AKIII, indicating that the genes are linked in the transformed lines.

The amino acid composition of the seeds from these lines was determined as described above. Again seeds  
 5 expressing Corynebacteria DHDPS protein showed increased levels of lysine. Expression of DHDPS alone resulted in 5% to 40% increases in total seed lysine. Expression of DHDPS along with AKIII-M4 results in lysine increases of more than 400%. A summary of all the different  
 10 transformed lines is shown in Table 3A

TABLE 3A

LINE-SEED	GUS + to -	DHDPS	AKIII	% LYS TOT
A2396-145	6 to 4	+	-	7.5
A2396-233	3 to 1	+	+	25
A2396-234	15 to 1	+	+	16
A2396-248	4 to 10	+	-	6.3
A2396-263	14 to 2	-	-	
A2396-240	7 to 1	+	+	11
A2396-267	2 to 53	+	+	8.9
A2242-273	11 to 5	+	+	13
A2242-315	6 to 2	+	+	16
A2242-316	1 to 15	+	+	12
A5403-175	7 to 3	+	+	7.6
A5403-181	7 to 3	-	-	5.7
A5403-183	6 to 4	+	+	6
A5403-185	9 to 11	+	+	7.6 P
A5403-196	9 to 1	+	+	7.6
A5403-203	6 to 36	+	+	6.1 P
A5403-204	17 to 3	+	+	8.8 P
A5403-212	13 to 5	+	+	9.4 P
A5403-214	21 to 16	+	+	32
A5403-216	14 to 4	+	-	8.2 P



A5403-218	13 to 9	+	+	9.8 P
A5403-222	12 to 27	+	+	15
A5403-225	14 to 12	+	+	13

P indicates seeds were pooled before meal extraction and assay

#### EXAMPLE 7

##### Isolation of a Plant

##### 5        Lysine Ketoglutarate Reductase Gene

Lysine Ketoglutarate Reductase (LKR) enzyme activity has been observed in immature endosperm of developing maize seeds [Arruda et al. (1982) Plant Physiol. 69:988-989]. LKR activity increases sharply from the onset of endosperm development, reaches a peak level at about 20 d after pollination, and then declines [Arruda et al. (1983) Phytochemistry 22:2687-2689].

In order to clone the corn LKR gene, RNA was isolated from developing seeds 19 d after pollination. This RNA was sent to Clontech Laboratories, Inc., (Palo Alto, CA) for the custom synthesis of a cDNA library in the vector Lambda Zap II. The conversion of the Lambda Zap II library into a phagemid library, then into a plasmid library was accomplished following the protocol provided by Clontech. Once converted into a plasmid library the ampicillin-resistant clones obtained carry the cDNA insert in the vector pBluescript SK(-). Expression of the cDNA is under control of the lacZ promoter on the vector.

Two phagemid libraries were generated using the mixtures of the Lambda Zap II phage and the filamentous helper phage of 100  $\mu$ L to 1  $\mu$ L. Two additional libraries were generated using mixtures of 100  $\mu$ L Lambda Zap II to 10  $\mu$ L helper phage and 20  $\mu$ L Lambda Zap II to 10  $\mu$ L helper phage. The titers of the phagemid preparations were similar regardless of the mixture used and were about  $2 \times 10^3$  ampicillin-resistant-

transfectants per mL with *E. coli* strain XL1-Blue as the host and about  $1 \times 10^3$  with DE126 (see below) as host.

To select clones that carried the LKR gene a specially designed *E. coli* host, DE126 was constructed. Construction of DE126 occurred in several stages.

(1) A generalized transducing stock of coliphage Plvir was produced by infection of a culture of TST1 [*F*<sup>-</sup>, *araD*139, *delta*(*argF-lac*)205, *flb*5301, *ptsF*25, *relA*1, *rpsL*150, *malE*52::*Tn10*, *deoC*1,  $\lambda$ <sup>-</sup>] (*E. coli* Genetic Stock Center #6137) using a standard method (for Methods see J. Miller, Experiments in Molecular Genetics).

(2) This phage stock was used as a donor in a transductional cross (for Method see J. Miller, Experiments in Molecular Genetics) with strain GIF106M1 [*F*<sup>-</sup>, *arg*<sup>-</sup>, *ilvA*296, *lysC*1001, *thrA*1101, *metL*1000,  $\lambda$ <sup>-</sup>, *rpsL*9, *malT*1, *xyl*-7, *mtl*-2, *thi*1(?), *supE*44(?)] (*E. coli* Genetic Stock Center #5074) as the recipient. Recombinants were selected on rich medium [L broth supplemented with DAP] containing the antibiotic tetracycline. The transposon *Tn10*, conferring tetracycline resistance, is inserted in the *malE* gene of strain TST1. Tetracycline-resistant transductants derived from this cross are likely to contain up to 2 min of the *E. coli* chromosome in the vicinity of *malE*. The genes *malE* and *lysC* are separated by less than 0.5 minutes, well within cotransduction distance.

(3) 200 tetracycline-resistant transductants were thoroughly phenotyped; appropriate fermentation and nutritional traits were scored. The recipient strain GIF106M1 is completely devoid of aspartokinase isozymes due to mutations in *thrA*, *metL* and *lysC*, and therefore requires the presence of threonine, methionine, lysine and meso-diaminopimelic acid (DAP) for growth. Transductants that had inherited *lysC*<sup>+</sup> with *malE*::*Tn10*

from TST1 would be expected to grow on a minimal medium that contains vitamin B1, L-arginine, L-isoleucine and L-valine in addition to glucose which serves as a carbon and energy source. Moreover strains having the genetic constitution of lysC<sup>+</sup>, metL<sup>-</sup> and thrA<sup>-</sup> will only express the lysine sensitive aspartokinase. Hence addition of lysine to the minimal medium should prevent the growth of the lysC<sup>+</sup> recombinant by leading to starvation for threonine, methionine and DAP. Of the 200 tetracycline resistant transductants examined, 49 grew on the minimal medium devoid of threonine, methionine and DAP. Moreover, all 49 were inhibited by the addition of L-lysine to the minimal medium. One of these transductants was designated DE125. DE125 has the phenotype of tetracycline resistance, growth requirements for arginine, isoleucine and valine, and sensitivity to lysine. The genotype of this strain is F<sup>-</sup> malE52::Tn10 arg<sup>-</sup> ilvA296 thrA1101 metL1000 lambda-rpsL9 malT1 xyl-7 mtl-2 thi1(?) supE44(?).

(4) This step involves production of a male derivative of strain DE125. Strain DE125 was mated with the male strain AB1528 [F'16/delta(gpt-proA)62, lacY1 or lacZ4, glnV44, galK2 rac<sup>-</sup>(?), hisG4, rfbD1, mgl-51, kdgK51(?), ilvC7, argE3, thi-1] (E. coli Genetic Stock Center #1528) by the method of conjugation. F'16 carries the ilvGMEDAYC gene cluster. The two strains were cross streaked on rich medium permissive for the growth of each strain. After incubation, the plate was replica plated to a synthetic medium containing tetracycline, arginine, vitamin B1 and glucose. DE125 cannot grow on this medium because it cannot synthesize isoleucine. Growth of AB1528 is prevented by the inclusion of the antibiotic tetracycline and the omission of proline and histidine from the synthetic medium. A patch of cells grew on this selective medium.

These recombinant cells underwent single colony isolation on the same medium. The phenotype of one clone was determined to be  $Ilv^+$ ,  $Arg^-$ ,  $Tet^R$ , Lysine-sensitive, male specific phage (MS2)-sensitive, consistent with the simple transfer of F'16 from AB1528 to DE125. This clone was designated DE126 and has the genotype F'16/malE52::Tn10,  $arg^-$ , ilvA296, thrA1101, metL100, lysC<sup>+</sup>,  $\lambda^-$ , rpsL9, maltT1, xyl-7, mtl-2, thi-1?, supE44?. It is inhibited by 20  $\mu$ g/mL of L-lysine in a synthetic medium.

To select for clones from the corn cDNA library that carried the LKR gene, 100  $\mu$ L of the phagemid library was mixed with 100  $\mu$ L of an overnight culture of DE126 grown in L broth and the cells were plated on synthetic media containing vitamin B1, L-arginine, 3% glucose as a carbon and energy source, 100  $\mu$ g/mL ampicillin and L-lysine at 20, 30 or 40  $\mu$ g/mL. Four plates at each of the three different lysine concentrations were prepared. The amount of phagemid and DE126 cells was expected to yield about  $1 \times 10^5$  ampicillin-resistant transfectants per plate. Ten to thirty lysine-resistant colonies grew per plate (about 1 lysine-resistant per 5000 ampicillin-resistant colonies).

Plasmid DNA was isolated from 10 independent clones and retransformed into DE126. Seven of the ten DNAs yielded lysine-resistant clones demonstrating that the lysine-resistance trait was carried on the plasmid. Several of the cloned DNAs were sequenced and biochemically characterized. The inserted DNA fragments were found to be derived from the E. coli genome, rather than a corn cDNA indicating that the cDNA library provided by Clontech was contaminated. A new cDNA library will therefore be prepared and screened as described above.

EXAMPLE 8Construction of Synthetic Genes  
in Expression Vector pSK5

To facilitate the construction and expression of  
5 the synthetic genes described below, it was necessary to  
construct a plasmid vector with the following  
attributes:

1. No Ear I restriction endonuclease sites  
such that insertion of sequences would produce a unique  
10 site.
2. Containing a tetracycline resistance gene  
to avoid loss of plasmid during growth and expression of  
toxic proteins.
3. Containing approximately 290 bp from  
15 plasmid pBT430 including the T7 promoter and terminator  
segment for expression of inserted sequences in E. coli.
4. Containing unique EcoR I and Nco I  
restriction endonuclease recognition sites in proper  
location behind the T7 promoter to allow insertion of  
20 the oligonucleotide sequences.

To obtain attributes 1 and 2 Applicants used  
plasmid pSK1 which was a spontaneous mutant of pBR322  
where the ampicillin gene and the Ear I site near that  
gene had been deleted. Plasmid pSK1 retained the  
25 tetracycline resistance gene, the unique EcoR I  
restriction sites at base 1 and a single Ear I site at  
base 2353. To remove the Ear I site at base 2353 of  
pSK1 a polymerase chain reaction (PCR) was performed  
using pSK1 as the template. Approximately 10 femtomoles  
30 of pSK1 were mixed with 1 µg each of oligonucleotides  
SM70 and SM71 which had been synthesized on an ABI1306B  
DNA synthesizer using the manufacturer's procedures.

SM70 5'-CTGACTCGCTGCGCTCGGTC 3' SEQ ID NO:16  
35 SM71 5'-TATTTTCTCCTTACGCATCTGTGC-3' SEQ ID NO:17

The priming sites of these oligonucleotides on the pSK1 template are depicted in Figure 7. The PCR was performed using a Perkin-Elmer Cetus kit (Emeryville, CA) according to the instructions of the vendor on a thermocycler manufactured by the same company. The 25 cycles were 1 min at 95°, 2 min at 42° and 12 min at 72°. The oligonucleotides were designed to prime replication of the entire pSK1 plasmid excluding a 30 b fragment around the Ear I site (see Figure 7). Ten microliters of the 100 µL reaction product were run on a 1% agarose gel and stained with ethidium bromide to reveal a band of about 3.0 kb corresponding to the predicted size of the replicated plasmid.

The remainder of the PCR reaction mix (90 µL) was mixed with 20 µL of 2.5 mM deoxynucleotide triphosphates (dATP, dTTP, dGTP, and dCTP), 30 units of Klenow enzyme added and the mixture incubated at 37° for 30 min followed by 65° for 10 min. The Klenow enzyme was used to fill in ragged ends generated by the PCR. The DNA was ethanol precipitated, washed with 70% ethanol, dried under vacuum and resuspended in water. The DNA was then treated with T4 DNA kinase in the presence of 1 mM ATP in kinase buffer. This mixture was incubated for 30 mins at 37° followed by 10 min at 65°. To 10 µL of the kinased preparation, 2 µL of 5X ligation buffer and 10 units of T4 DNA ligase were added. The ligation was carried out at 15° for 16 h. Following ligation, the DNA was divided in half and one half digested with Ear I enzyme. The Klenow, kinase, ligation and restriction endonuclease reactions were performed as described in Sambrook et al., [Molecular Cloning, A Laboratory Manual, 2nd ed. (1989) Cold Spring Harbor Laboratory Press]. Klenow, kinase, ligase and most restriction endonucleases were purchased from BRL. Some restriction endonucleases were purchased from NEN Biolabs (Beverly,

MA) or Boehringer Mannheim (Indianapolis, IN). Both the ligated DNA samples were transformed separately into competent JM103 [supE thi del (lac-proAB) F' [traD36 porAB, lacIq lacZ del M15] restriction minus] cells using the CaCl<sub>2</sub> method as described in Sambrook et al., [Molecular Cloning, A Laboratory Manual, 2nd ed. (1989) Cold Spring Harbor Laboratory Press] and plated onto media containing 12.5 ug/mL tetracycline. With or without Ear I digestion the same number of transformants were recovered suggesting that the Ear I site had been removed from these constructs. Clones were screened by preparing DNA by the alkaline lysis miniprep procedure as described in Sambrook et al., [Molecular Cloning, A Laboratory Manual, 2nd ed. (1989) Cold Spring Harbor Laboratory Press] followed by restriction endonuclease digest analysis. A single clone was chosen which was tetracycline-resistant and did not contain any Ear I sites. This vector was designated pSK2. The remaining EcoR I site of pSK2 was destroyed by digesting the plasmid with EcoR I to completion, filling in the ends with Klenow and ligating. A clone which did not contain an EcoR I site was designated pSK3.

To obtain attributes 3 and 4 above, the bacteriophage T7 RNA polymerase promoter/terminator segment from plasmid pBT430 (see Example 2) was amplified by PCR. Oligonucleotide primers SM78 (SEQ ID NO:18) and SM79 (SEQ ID NO:19) were designed to prime a 300b fragment from pBT430 spanning the T7 promoter/terminator sequences (see Figure 7).

SM78 5'-TTCATCGATAGGCGACCACACCCGTCC-3' SEQ ID NO:18

SM79 5'-AATATCGATGCCACGATGCGTCCGGCG-3' SEQ ID NO:19

The PCR reaction was carried out as described previously using pBT430 as the template and a 300 bp

fragment was generated. The ends of the fragment were filled in using Klenow enzyme and kinased as described above. DNA from plasmid pSK3 was digested to completion with PvuII enzyme and then treated with calf intestinal alkaline phosphatase (Boehringer Mannheim) to remove the 5' phosphate. The procedure was as described in Sambrook et al., [Molecular Cloning, A Laboratory Manual, 2nd ed. (1989) Cold Spring Harbor Laboratory Press]. The cut and phosphatased pSK3 DNA was purified by ethanol precipitation and a portion used in a ligation reaction with the PCR generated fragment containing the T7 promoter sequence. The ligation mix was transformed into JM103 [supE thi del (lac-proAB) F' [traD36 porAB, lacIq lacZ del M15] restriction minus] and tetracycline-resistant colonies were screened. Plasmid DNA was prepared via the alkaline lysis mini-prep method and restriction endonuclease analysis was performed to detect insertion and orientation of the PCR product. Two clones were chosen for sequence analysis: Plasmid pSK5 had the fragment in the orientation shown in Figure 7. Sequence analysis performed on alkaline denatured double-stranded DNA using Sequenase® T7 DNA polymerase (US Biochemical Corp) and manufacturer's suggested protocol revealed that pSK5 had no PCR replication errors within the T7 promoter/terminator sequence.

The strategy for the construction of repeated synthetic gene sequences based on the Ear I site is depicted in Figure 8. The first step was the insertion of an oligonucleotide sequence encoding a base gene of 14 amino acids. This oligonucleotide insert contained a unique Ear I restriction site for subsequent insertion of oligonucleotides encoding one or more heptad repeats and added an unique Asp 718 restriction site for use in transfer of gene sequences to plant vectors. The



overhanging ends of the oligonucleotide set allowed insertion into the unique Nco I and EcoR I sites of vector pSK5.

5  
 SM81 M E E K M K A M E E K  
 5'-CATGGAGGAGAAGATGAAGGCGATGGAAGAGAAG  
 SM80 3'-CTCCTCTTCTACTTCGCTACCTTCTCTTC  
 NCO I EAR I  
 10 M K A (SEQ ID NO:22)  
 SM81 ATGAAGGCGTGATAGGTACCG-3' (SEQ ID NO:20)  
 SM80 TACTTCCGCACTATCCATGGCTTAA-5' (SEQ ID NO:21)  
 ASP718 ECOR I

15 DNA from plasmid pSK5 was digested to completion with Nco I and EcoR I restriction endonucleases and purified by agarose gel electrophoresis. Purified DNA (0.1 ug) was mixed with 1 µg of each oligonucleotide SM80 (SEQ ID NO:14) and SM81 (SEQ ID NO:13) and ligated.  
 20 The ligation mixture was transformed into *E. coli* strain JM103 [supE thi del (lac-proAB) F' [traD36 porAB, lacIq lacZ del M15] restriction minus] and tetracycline resistant transformants screened by rapid plasmid DNA preps followed by restriction digest analysis. A clone  
 25 was chosen which had one each of Ear I, Nco I, Asp 718 and EcoR I sites indicating proper insertion of the oligonucleotides. This clone was designated pSK6 (Figure 9). Sequencing of the region of DNA following the T7 promoter confirmed insertion of oligonucleotides  
 30 of the expected sequence.

Repetitive heptad coding sequences were added to the base gene construct of described above by generating oligonucleotide pairs which could be directly ligated into the unique Ear I site of the base gene. Oligo-  
 35 nucleotides SM84 (SEQ ID NO:23) and SM85 (SEQ ID NO:24) code for repeats of the SSP5 heptad. Oligonucleotides

SM82 (SEQ ID NO:25) and SM83 (SEQ ID NO:26) code for repeats of the SSP7 heptad.

	SSP5	M E E K M K A	(SEQ ID NO:28)
5	SM84	5'-GATGGAGGAGAAGATGAAGGC-3'	(SEQ ID NO:23)
	SM85	3'- CCTCCTCTTCTACTCCGCTA-5'	(SEQ ID NO:24)
	SSP7	M E E K L K A	(SEQ ID NO:27)
	SM82	5'-GATGGAGGAGAAGCTGAAGGC-3'	(SEQ ID NO:25)
10	SM83	3'- CCTCCTCTTCGACTTCGCTA-5'	(SEQ ID NO:26)

Oligonucleotide sets were ligated and purified to obtain DNA fragments encoding multiple heptad repeats for insertion into the expression vector. Oligonucleotides from each set totalling about 2 µg were kinased, and ligated for 2 h at room temperature. The ligated multimers of the oligonucleotide sets were separated on an 18% non-denaturing 20 X 20 X 0.015 cm polyacrylamide gel (Acrylamide: bis-acrylamide = 19:1). Multimeric forms which separated on the gel as 168 bp (8n) or larger were purified by cutting a small piece of polyacrylamide containing the band into fine pieces, adding 1.0 mL of 0.5 M ammonium acetate, 1 mM EDTA (pH 7.5) and rotating the tube at 37° overnight. The polyacrylamide was spun down by centrifugation, 1 µg of tRNA was added to the supernatant, the DNA fragments were precipitated with 2 volumes of ethanol at -70°, washed with 70% ethanol, dried, and resuspended in 10 µL of water.

Ten micrograms of pSK6 DNA were digested to completion with Ear I enzyme and treated with calf intestinal alkaline phosphatase. The cut and phosphatased vector DNA was isolated following electrophoresis in a low melting point agarose gel by cutting out the banded DNA, liquifying the agarose at 55°, and purifying over NACS PREPAC™ columns (BRL)

following manufacturer's suggested procedures. Approximately 0.1 µg of purified Ear I digested and phosphatase treated pSK6 DNA was mixed with 5 µL of the gel purified multimeric oligonucleotide sets and

5 ligated. The ligated mixture was transformed into *E. coli* strain JM103 [supE thi del (lac-proAB) F' [traD36 porAB, lacIq lacZ del M15] restriction minus] and tetracycline-resistant colonies selected. Clones

10 were screened by restriction digests of rapid plasmid prep DNA to determine the length of the inserted DNA. Restriction endonuclease analyses were usually carried out by digesting the plasmid DNAs with Asp 718 and

15 Bgl II, followed by separation of fragments on 18% non-denaturing polyacrylamide gels. Visualization of fragments with ethidium bromide, showed that a 150 bp fragment was generated when only the base gene segment was present. Inserts of the oligonucleotide fragments increased this size by multiples of 21 bases. From this

20 screening several clones were chosen for DNA sequence analysis and expression of coded sequences in *E. coli*. The first and last SSP5 heptads flanking the sequence of each construct are from the base gene described above. Inserts are designated by underlining (Table 4).

Table 4  
Sequence by Heptad

<u>Clone #</u>	<u>SEO ID NO:</u>	<u>Amino Acid Repeat (SSP)</u>	<u>SEO ID NO:</u>
C15	29	5. <u>7.7.7.7.7.7.5</u>	30
C20	31	5. <u>7.7.7.7.7.7.5</u>	32
C30	33	5. <u>7.7.7.7.7.7.5</u>	34
D16	35	5. <u>5.5.5.5</u>	36
D20	37	5. <u>5.5.5.5.5</u>	38
25 D33	39	5. <u>5.5.5.5</u>	40

Because the gel purification of the oligomeric forms of the oligonucleotides did not give the expected

enrichment of longer (i.e., >8n) inserts, Applicants used a different procedure for a subsequent round of insertion constructions. For this series of constructs four more sets of oligonucleotides were generated which  
 5 code for SSP 8,9,10 and 11 amino acid sequences respectively:

	SSP8	M E E K L K K	(SEQ ID NO:49)
	SM86	5'-GATGGAGGAGAAGCTGAAGAA-3'	(SEQ ID NO:41)
10	SM87	3'- CCTCCTCTTCGACTTCTTCTA-5'	(SEQ ID NO:42)
	SSP9	M E E K L K W	(SEQ ID NO:50)
	SM88	5'-GATGGAGGAGAAGCTGAAGTG-3'	(SEQ ID NO:43)
15	SM89	3'- CCTCCTCTTCGACTTCACCTA-5'	(SEQ ID NO:44)
	SSP10	M E E K M K K	(SEQ ID NO:51)
	SM90	5'-GATGGAGGAGAAGATGAAGAA-3'	(SEQ ID NO:45)
	SM91	3'- CCTCCTCTTCTACTTCTTCTA-5'	(SEQ ID NO:46)
20	SSP11	M E E K M K W	(SEQ ID NO:52)
	SM92	5'-GATGGAGGAGAAGATGAAGTG-3'	(SEQ ID NO:47)
	SM93	3'- CCTCCTCTTCTACTTCACCTA-5'	(SEQ ID NO:48)

The following HPLC procedure was used to purify  
 25 multimeric forms of the oligonucleotide sets after  
 kinasing and ligating the oligonucleotides as described  
 above. Chromatography was performed on a Hewlett  
 Packard Liquid Chromatograph instrument, Model 1090M.  
 Effluent absorbance was monitored at 260 nm. Ligated  
 30 oligonucleotides were centrifuged at 12,000xg for 5 min  
 and injected onto a 2.5  $\mu$  TSK DEAE-NPR ion exchange  
 column (35 cm x 4.6 mm I.D.) fitted with a 0.5  $\mu$  in-line  
 filter (Supelco). The oligonucleotides were separated  
 on the basis of length using a gradient elution and a  
 35 two buffer mobile phase [Buffer A: 25 mM Tris-Cl, pH  
 9.0, and Buffer B: Buffer A + 1 M NaCl]. Both Buffers  
 A and B were passed through 0.2  $\mu$  filters before use.

The following gradient program was used with a flow rate of 1 mL per min at 30°:

<u>Time</u>	<u>%A</u>	<u>%B</u>
initial	75	25
0.5 min	55	45
5 min	50	50
20 min	38	62
23 min	0	100
30 min	0	100
31 min	75	25

- 5 Fractions (500 µL) were collected between 3 min and 9 min. Fractions corresponding to lengths between 120 bp and 2000 bp were pooled as determined from control separations of restriction digests of plasmid DNAs.
- 10 The 4.5 mL of pooled fractions for each oligo-nucleotide set were precipitated by adding 10 µg of tRNA and 9.0 mL of ethanol, rinsed twice with 70% ethanol and resuspended in 50 µL of water. Ten microliters of the resuspended HPLC purified oligonucleotides were added to
- 15 0.1 µg of the Ear I cut, phosphatased pSK6 DNA described above and ligated overnight at 15°. All six oligo-nucleotide sets described above which had been kinased and self-ligated but not purified by gel or HPLC were also used in separate ligation reactions with the pSK6
- 20 vector. The ligation mixtures were transformed into *E. coli* strain DH5α [supE44 del lacU169 (phi 80 lacZ del M15) hsdR17 recA1 endA1 gyr196 thi1 relA1] and tetracycline-resistant colonies selected. Applicants chose to use the DH5α [supE44 del lacU169 (phi 80 lacZ
- 25 del M15) hsdR17 recA1 endA1 gyr196 thi1 relA1] strain for all subsequent work because this strain has a very high transformation rate and is recA-. The recA- phenotype eliminates concerns that these repetitive DNA

structures may be substrates for homologous recombination leading to deletion of multimeric sequences.

Clones were screened as described above. Several clones were chosen to represent insertions of each of the six oligonucleotide sets. The first and last SSP5 heptads flanking the sequence represent the base gene sequence. Insert sequences are underlined. Clone numbers including the letter "H" designate HPLC-purified oligonucleotides (Table 5).

Table 5

Clone #	SEO ID NO:	Sequence by Heptad	
		Amino Acid Repeat (SSP)	SEO ID NO:
82-4	53	<u>7.7.7.7.7.7.5</u>	54
84-H3	55	5. <u>5.5.5</u>	56
86-H23	57	5. <u>8.8.8</u>	58
88-2	59	5. <u>9.9.9.5</u>	60
90-H8	61	5. <u>10.10.10.5</u>	62
92-2	63	5. <u>11.11.11.5</u>	64

The loss of the first base gene repeat in clone 82-4 may have resulted from homologous recombination between the base gene repeats 5.5 before the vector pSK6 was transferred to the recA- strain. The HPLC procedure did not enhance insertion of longer multimeric forms of the oligonucleotide sets into the base gene but did serve as an efficient purification of the ligated oligonucleotides.

Oligonucleotides were designed which coded for mixtures of the SSP sequences and which varied codon usage as much as possible. This was done to reduce the possibility of deletion of repetitive inserts by recombination once the synthetic genes were transformed into plants and to extend the length of the constructed gene segments. These oligonucleotides encode four

repeats of heptad coding units (28 amino acid residues) and can be inserted at the unique Ear I site in any of the previously constructed clones. SM96 and SM97 code for SSP(5)<sub>4</sub>, SM98 and SM99 code for SSP(7)<sub>4</sub> and SM100 plus SM101 code for SSP8.9.8.9.

```

          M E E K M K A M E E K M K
SM96      5'-GATGGAGGAAAAGATGAAGCGATGGAGGAGAAAATGAAA
SM97      3'   CCTCCTTTTCTACTTCCGCTACCTCCTCTTTTACTTT
10  A M E E K M K A M E E K M K A      (SEQ ID NO:67)
GCTATGGAGGAAAAGATGAAGCGATGGAGGAGAAAATGAAGGC-3'      (SEQ ID NO:65)
CGATACCTCCTTTTCTACTTTTCGCTACCTCCTCTTTTACTTCCGCTA-5'      (SEQ ID NO:66)

          M E E K L K A M E E K L K
15  SM98      5'-GATGGAGGAAAAGCTGAAAGCGATGGAGGAGAAACTCAAG
SM99      3'   CCTCCTTTTTCGACTTTTCGCTACCTCCTCTTTGAGTTT
A M E E K L K A M E E K L K A      (SEQ ID NO:70)
GCTATGGAAGAAAAGCTTAAAGCGATGGAGGAGAAACTGAAGGC-3'      (SEQ ID NO:68)
CGATACCTTCTTTTTCGAATTTTCGCATCCTCCTCTTTGACTTCCGCTA-5'      (SEQ ID NO:69)
20

          M E E K L K K M E E K L K
SM100     5'-GATGGAGGAAAAGCTTAAAGATGGAAGAAAAGCTGAAA
SM101     3'   CCTCCTTTTTCGAATTTCTTCTACCTTCTTTTTCGACTTT
W M E E K L K K M E E K L K W      (SEQ ID NO:73)
25  TGGATGGAGGAGAAACTCAAAAAGATGGAGGAAAAGCTTAAATG-3'      (SEQ ID NO:71)
ACCTACCTCCTCTTTGAGTTTTTTCATCCTCCTTTTTCGAATTTACCTA-5'      (SEQ ID NO:72)

```

DNA from clones 82-4 and 84-H3 were digested to completion with Ear I enzyme, treated with phosphatase and gel purified. About 0.2 µg of this DNA were mixed with 1.0 µg of each of the oligonucleotide sets SM96 and SM97, SM98 and SM99 or SM100 and SM101 which had been previously kinased. The DNA and oligonucleotides were ligated overnight and then the ligation mixes transformed into *E. coli* strain DH5α. Tetracycline-resistant colonies were screened as described above for the presence of the oligonucleotide inserts. Clones

were chosen for sequence analysis based on their restriction endonuclease digestion patterns (Table 6).

Table 6

Clone #	SEQ ID NO:	Sequence by Heptad	
		Amino Acid Repeat (SSP)	SEQ ID NO:
2-9	74	7.7.7.7.7.7.8.9.8.9.5	75
3-5	78	7.7.7.7.7.7.5.5	79
5-1	76	5.5.5.7.7.7.7.5	77

Inserted oligonucleotide segments are underlined

- 5 Clone 2-9 was derived from oligonucleotides SM100 (SEQ ID NO:71) and SM101 (SEQ ID NO:72) ligated into the Ear I site of clone 82-4 (see above). Clone 3-5 (SEQ ID NO:78) was derived from the insertion of the first 22 bases of the oligonucleotide set SM96 (SEQ ID NO:65) and
- 10 SM97 (SEQ ID NO:66) into the Ear I site of clone 82-4 (SEQ ID NO:53). This partial insertion may reflect improper annealing of these highly repetitive oligos. Clone 5-1 (SEQ ID NO:76) was derived from oligo-
- 15 nucleotides SM98 (SEQ ID NO:68) and SM99 (SEQ ID NO:69) ligated into the Ear I site of clone 84-H3 (SEQ ID NO:55) (see section).

#### Strategy II.

- 20 A second strategy for construction of synthetic gene sequences was implemented to allow more flexibility in both DNA and amino acid sequence. This strategy is depicted in Figure 10 and Figure 11. The first step was the insertion of an oligonucleotide sequence encoding a base gene of 16 amino acids into the original vector
- 25 pSK5. This oligonucleotide insert contained an unique Ear I site as in the previous base gene construct for use in subsequent insertion of oligonucleotides encoding one or more heptad repeats. The base gene also included a BspH I site at the 3' terminus. The overhanging ends



of this cleavage site are designed to allow "in frame" protein fusions using Nco I overhanging ends.

Therefore, gene segments can be multiplied using the duplication scheme described in Figure 11. The overhanging ends of the oligonucleotide set allowed insertion into the unique Nco I and EcoR I sites of vector pSK5.

```

10      M E E K M K K L E E K
SM107   5'-CATGGAGGAGAAGATGAAAAAGCTCGAAGAGAAG
SM106   3'-CTCCTCTTCTACTTTTTCGAGCTTCTCTTC
        NCO I                               EAR I

        M K V M K                               (SEQ ID NO:82)
15      ATGAAGGTCATGAAGTGATAGGTACCG-3'         (SEQ ID NO:80)
        TACTTCCAGTACTTCACTATCCATGGCTTAA-5' (SEQ ID NO:81)
        BSPH I  ASP 718

```

The oligonucleotide set was inserted into pSK5 vector as described in Strategy I above. The resultant plasmid was designated pSK34.

Oligonucleotide sets encoding 35 amino acid "segments" were ligated into the unique Ear I site of the pSK34 base gene using procedures as described above. In this case, the oligonucleotides were not gel or HPLC purified but simply annealed and used in the ligation reactions. The following oligonucleotide sets were used:

```

30      SEG 3      L E E K M K A M E D K M K W
SM110   5'-GCTGGAAGAAAAGATGAAGGCTATGGAGGACAAGATGAAATGG
SM111   3'-CCTTCTTTTCTACTTCCGATACCTCCTGTTCTACTTTACC

        L E E K M K K                               (SEQ ID NO:85)
35                                             (amino acids 8-28)
        CTTGAGGAAAAGATGAAGAA-3'                     (SEQ ID NO:83)
        GAACTCCTTTTCTACTTCTTCGA-5'                   (SEQ ID NO:84)

```

5 L E E K M K K (SEQ ID NO:86)  
(amino acids 8-28)  
CTTGAGGAGAAAATGAAGAA-3' (SEQ ID NO:87)  
GAACTCCTCTTTTACTTCTTCGA-5' (SEQ ID NO:88)

10 SEG 5 L K E E M A K M K D E M W K  
SM114 5'-GCTCAAGGAGGAAATGGCTAAGATGAAAGACGAAATCTGGAAA  
SM115 3'-GTTCTCTCTTTACCGATTCTACTTTCTGCTTTACACCTTT

15 L K E E M K K (SEQ ID NO:89)  
(amino acids 8-28)  
CTGAAAGAGGAAATGAAGAA (SEQ ID NO:90)  
GACTTTCTCCTTTACTTCTTCGA (SEQ ID NO:91)

Clones were screened for the presence of the inserted segments by restriction digestion followed by separation of fragments on 6% acrylamide gels. Correct insertion of oligonucleotides was confirmed by DNA sequence analyses. Clones containing segments 3, 4 and 5 respectively were designated pSKseg3, pSKseg4, and pSKseg5.

These "segment" clones were used in a duplication scheme as shown in Figure 11. Ten  $\mu$ g of plasmid pSKseg3 were digested to completion with Nhe I and BspH I and the 1503 bp fragment isolated from an agarose gel using the Whatmann paper technique. Ten  $\mu$ g of plasmid pSKseg4 were digested to completion with Nhe I and Nco I and the 2109 bp band gel isolated. Equal amounts of these fragments were ligated and recombinants selected on tetracycline. Clones were screened by restriction digestions and their sequences confirmed. The resultant plasmid was designated pSKseg34.

pSKseg34 and pSKseg5 plasmid DNAs were digested, fragments isolated and ligated in a similar manner as above to create a plasmid containing DNA sequences encoding segment 5 fused to segments 3 and 4. This construct was designated pSKseg534 and encodes the following amino acid sequence:

SSP534 NH<sub>2</sub>-MEEKMKKLKEEMAKMKDEMWKLKEEMKKLEEKMKVMEEKMKKLEEKMKKA  
MEDKMKWLEEKMKKLEEKMKVMEEKMKKLEEKMKAMEDKMKWLEEKMKK  
LEEKMKVMK-COOH (SEQ ID NO:92)

### EXAMPLE 9

#### Construction of SSP Chimeric Genes for Expression in the Seeds of Plants

To express the synthetic gene products described in Example 8 in plant seeds, the sequences were transferred to the seed promoter vectors CW108, CW109 or ML113 (Figure 12). The vectors CW108 and ML113 contain the bean phaseolin promoter (from base +1 to base -494), and 1191 bases of the 3' sequences from bean phaseolin gene. CW109 contains the soybean  $\beta$ -conglycinin promoter (from base +1 to base -619) and the same 1191 bases of 3' sequences from the bean phaseolin gene. These vectors were designed to allow direct cloning of coding sequences into unique Nco I and Asp 718 sites. These vectors also provide sites (Hind III or Sal I) at the 5' and 3' ends to allow transfer of the promoter/coding region/ 3' sequences directly to appropriate binary vectors.

To insert the synthetic storage protein gene sequences, 10  $\mu$ g of vector DNA were digested to completion with Asp 718 and Nco I restriction endonucleases. The linearized vector was purified via electrophoresis on a 1.0% agarose gel overnight electrophoresis at 15 volts. The fragment was collected by cutting the agarose in front of the band, inserting a

10 X 5 mm piece of Whatman 3MM paper into the agarose and electrophoresing the fragment into the paper [Errington, (1990) Nucleic Acids Research, 18:17]. The fragment and buffer were spun out of the paper by  
5 centrifugation and the DNA in the ~100  $\mu$ L was precipitated by adding 10 mg of tRNA, 10  $\mu$ L of 3 M sodium acetate and 200  $\mu$ L of ethanol. The precipitated DNA was washed twice with 70% ethanol and dried under vacuum. The fragment DNA was resuspended in 20  $\mu$ L of  
10 water and a portion diluted 10-fold for use in ligation reactions.

Plasmid DNA (10 mg) from clones 3-5 and pSK534 was digested to completion with Asp 718 and Nco I restriction endonucleases. The digestion products were  
15 separated on an 18% polyacrylamide non-denaturing gel as described in Example 8. Gel slices containing the desired fragments were cut from the gel and purified by inserting the gel slices into a 1% agarose gel and electrophoresing for 20 min at 100 volts. DNA fragments  
20 were collected on 10 X 5 mm pieces of Whatman 3MM paper, the buffer and fragments spun out by centrifugation and the DNA precipitated with ethanol. The fragments were resuspended in 6  $\mu$ L water. One microliter of the diluted vector fragment described above, 2  $\mu$ L of 5X  
25 ligation buffer and 1  $\mu$ L of T4 DNA ligase were added. The mixture was ligated overnight at 15°.

The ligation mixes were transformed into *E. coli* strain DH5a [supE44 del lacU169 (phi 80 lacZ del M15) hsdR17 recA1 endA1 gyr196 thi1 relA1] and ampicillin-  
30 resistant colonies selected. The clones were screened by restriction endonuclease digestion analyses of rapid plasmid DNAs and by DNA sequencing.

EXAMPLE 10Tobacco Plants Containing the Chimeric GenesPhaseolin Promoter/cts/ecodapA,Phaseolin Promoter/cts/lysC-M4 and5  $\beta$ -conglycinin promoter/SSP3-5

The binary vector pZS97 was used to transfer the chimeric SSP3-5 gene of Example 9 and the chimeric *E. coli* dapA and lysC-M4 genes of Example 4 to tobacco plants. Binary vector pZS97 (Figure 13) is part of a  
10 binary Ti plasmid vector system [Bevan, (1984) Nucl. Acids. Res. 12:8711-8720] of Agrobacterium tumefaciens. The vector contains: (1) the chimeric gene nopaline synthase::neomycin phosphotransferase (nos::NPTII) as a selectable marker for transformed plant cells [Bevan et  
15 al., (1983) Nature 304:184-186], (2) the left and right borders of the T-DNA of the Ti plasmid [Bevan, (1984) Nucl. Acids. Res. 12:8711-8720], (3) the *E. coli* lacZ  $\alpha$ -complementing segment [Viering et al., (1982) Gene 19:259-267] with a unique Sal I site (pSK97K) or unique  
20 Hind III site (pZS97) in the polylinker region, (4) the bacterial replication origin from the Pseudomonas plasmid pVS1 [Itoh et al., (1984) Plasmid 11:206-220], and (5) the bacterial  $\beta$ -lactamase gene as a selectable marker for transformed *A. tumefaciens*.

25 Plasmid pZS97 DNA was digested to completion with Hind III enzyme and the digested plasmid was gel purified. The Hind III digested pZS97 DNA was mixed with the Hind III digested and gel isolated chimeric gene fragments, ligated, transformed as above and  
30 colonies selected on ampicillin.

Binary vectors containing the chimeric genes were transferred by tri-parental matings [Ruvkin et al., (1981) Nature 289:85-88] to Agrobacterium strain LBA4404/pAL4404 [Hockema et al., (1983), Nature  
35 303:179-180] selecting for carbenicillin resistance.

Cultures of Agrobacterium containing the binary vector were used to transform tobacco leaf disks [Horsch et al., (1985) Science 227:1229-1231]. Transgenic plants were regenerated in selective medium containing

5 kanamycin.

Transformed tobacco plants containing the chimeric gene,  $\beta$ -conglycinin promoter/SSP3-5/phaseolin 3' region, were thus obtained. Two transformed lines, pSK44-3A and pSK44-9A, which carried a single site insertion of the

10 SSP3-5 gene were identified based upon 3:1 segregation of the marker gene for kanamycin resistance. Progeny of the primary transformants, which were homozygous for the transgene, pSK44-3A-6 and pSK44-9A-5, were then

15 identified based upon 4:0 segregation of the kanamycin resistance in seeds of these plants.

Similarly, transformed tobacco plants with the chimeric genes phaseolin 5' region/cts/lysC-M4/phaseolin 3' region and phaseolin 5' region/cts/ecodapA/phaseolin 3' region were obtained. A transformed line, BT570-45A,

20 which carried a single site insertion of the DHDPS and AK genes was identified based upon 3:1 segregation of the marker gene for kanamycin resistance. Progeny from the primary transformant which were homozygous for the transgene, BT570-45A-3 and BT570-45A-4, were then

25 identified based upon 4:0 segregation of the kanamycin resistance in seeds of these plants.

To generate plants carrying all three chimeric genes genetic crosses were performed using the homozygous parents. Plants were grown to maturity in

30 greenhouse conditions. Flowers to be used as male and female were selected one day before opening and older flowers on the inflorescence removed. For crossing, female flowers were chosen at the point just before opening when the anthers were not dehiscent. The

35 corolla was opened on one side and the anthers removed.

Male flowers were chosen as flowers which had opened on the same day and had dehiscent anthers shedding mature pollen. The anthers were removed and used to pollinate the pistils of the anther-stripped female flowers. The pistils were then covered with plastic tubing to prevent further pollination. The seed pods were allowed to develop and dry for 4-6 weeks and harvested. Two to three separate pods were recovered from each cross. The following crosses were performed:

10

Male	X	Female
BT570-45A-3		pSK44-3A-6
BT570-45A-4		pSK44-3A-6
pSK44-3A-6		BT570-45A-4
BT570-45A-5		pSK44-9A-5
pSk44-9A-5		BT570-45A-5

15

Dried seed pods were broken open and seeds collected and pooled from each cross. Thirty seeds were counted out for each cross and for controls seeds from selfed flowers of each parent were used. Duplicate seed samples were hydrolyzed and assayed for total amino acid content as described in Example 5. The amount of increase in lysine as a percent of total seeds amino acids over wild type seeds, which contain 2.56% lysine, is presented in Table 7 along the copy number of each gene in the endosperm of the seed.

20

25

TABLE 7

male	X female	endosperm copy number AK & DHDPS genes	endosperm copy number SSP gene	lysine increase
BT570-45A	X BT570-45A	1.5*	0	0
pSK44-9A	X pSK44-9A	0	1.5*	0.12
pSK44-9A-5	X pSK44-9A-5	0	3.0	0.29
pSK44-9A-5	X BT570-45A-5	2	1	0.6

BT570-45A-5 X pSK44-9A-5	1	2	0.29
pSK44-3A X pSK44-3A	0	1.5*	0.28
pSK44-3A-6 X pSK44-3A-6	0	3.0	0.5
pSK44-3A-6 X BT570-45A-4	2	1	0.62
BT570-45A-3 X pSK44-3A-6	1	2	0.27
BT570-45A-4 X pSK44-3A-6	1	2	0.29

\* copy number is average in population of seeds

The results of these crosses demonstrate that the total lysine levels in seeds can be increased 10-25 percent by the coordinate expression of the lysine biosynthesis genes and the high lysine protein SSP3-5. In seeds derived from hybrid plants, this synergism is strongest when the biosynthesis genes are derived from the female parent, possibly due to gene dosage in the endosperm. It is expected that the lysine level would be further increased if the biosynthesis genes and the lysine-rich protein genes were all homozygous.

#### EXAMPLE 11

##### Soybean Plants Containing the Chimeric Genes

##### Phaseolin Promoter/cts/cordapa and

##### Phaseolin Promoter/SSP3-5

Transformed soybean plants that express the chimeric gene, phaseolin promoter/cts/cordapa/ phaseolin 3' region have been described in Example 6. Transformed soybean plants that express the chimeric gene, phaseolin promoter/SSP3-5/phaseolin 3' region, were obtained by inserting the chimeric gene as an isolated Hind III fragment into an equivalent soybean transformation vector plasmid pML63 (Figure 14 Example 6) and carrying out transformation as described in Example 6.

Seeds from primary transformants were sampled by cutting small chips from the sides of the seeds away from the embryonic axis. The chips were assayed for GUS activity as described in Example 6 to determine which of



the segregating seeds carried the transgenes. Half seeds were ground to meal and assayed for expression of SSP3-5 protein by Enzyme Linked ImmunoSorbent Assay (ELISA). Elisa was performed as follows:

- 5        A fusion protein of glutathione-S-transferase and the SSP3-5 gene product was generated through the use of the Pharmacia™ pGEX GST Gene Fusion System (Current Protocols in Molecular Biology, Vol. 2, pp 16.7.1-8, (1989) John Wiley and Sons). The fusion protein was
- 10       purified by affinity chromatography on glutathione agarose (Sigma) or glutathione sepharose (Pharmacia) beads, concentrated using Centricon 10™ (Amicon) filters, and then subjected to SDS polyacrylamide electrophoresis (15% Acrylamide, 19:1 Acrylamide:Bis-
- 15       acrylamide) for further purification. The gel was stained with Coomassie Blue for 30 min, destained in 50% Methanol, 10% Acetic Acid and the protein bands electroeluted using an Amicon™ Centiluter
- 20       Microelectroeluter (Paul T. Matsudaira ed., A Practical Guide to Protein and Peptide Purification for Microsequencing, Academic Press, Inc. New York, 1989). A second gel prepared and run in the same manner was stained in a non acetic acid containing stain [9 parts
- 25       0.1% Coomassie Blue G250 (Bio-Rad) in 50% methanol and 1 part Serva Blue (Serva, Westbury, NY) in distilled water] for 1-2 h. The gel was briefly destained in 20% methanol, 3% glycerol for 0.5-1 h until the GST-SSP3-5 band was just barely visible. This band was excised from the gel and sent with the electroeluted material to
- 30       Hazelton Laboratories for use as an antigen in immunizing a New Zealand Rabbit. A total of 1 mg of antigen was used (0.8 mg in gel, 0.2 mg in solution). Test bleeds were provided by Hazelton Laboratories every three weeks. The approximate titer was tested by
- 35       western blotting of E. coli extracts from cells

containing the SSP-3-5 gene under the control of the T7 promoter at different dilutions of protein and of serum.

IgG was isolated from the serum using a Protein A sepharose column. The IgG was coated onto microtiter  
5 plates at 5 µg per well. A separate portion of the IgG was biotinylated.

Aqueous extracts from transgenic plants were diluted and loaded into the wells usually starting with a sample containing 1 µg of total protein. The sample  
10 was diluted several more times to insure that at least one of the dilutions gave a result that was within the range of a standard curve generated on the same plate. The standard curve was generated using chemically synthesized SSP3-5 protein. The samples were incubated  
15 for one hour at 37° and the plates washed. The biotinylated IgG was then added to the wells. The plate was incubated at 37° for 1 hr and washed. Alkaline phosphatase conjugated to streptavidin was added to the wells, incubated at 37° for 1 hr and washed. A  
20 substrate consisting of 1 mg/ml p nitrophenylphosphate in 1M diethanolamine was added to the wells and the plates incubated at 37° for 1 hr. A 5% EDTA stop solution was added to the wells and the absorbance read at 405 nm minus 650 nm reading. Transgenic soybean  
25 seeds contained 0.5 to 2.0% of water extractable protein as SSP3-5.

The remaining half seeds positive for GUS and SSP3-5 protein were planted and grown to maturity in greenhouse conditions. To determine homozygotes for the  
30 GUS phenotype, seed from these R1 plants were screened for segregation of GUS activity as above. Plants homozygous for the phaseolin/SSP3-5 gene were crossed with homozygous transgenic soybeans expressing the *Corynebacterium* dapA gene product.

As an preferred alternative to bringing the chimeric SSP gene and chimeric cordapA geneA together via genetic crossing a single soybean tranformation vector carrying both genes was constructed. Plasmid pML63 carrying the chimeric gene phaseolin promoter/SSP3-5/phaseolin 3' region described above was cleaved with restriction enzyme BamH I and the BamH I fragment carrying the chimeric gene phaseolin promoter/cts/cordapA/ phaseolin 3' region (Example 5) was inserted. This vector can be transformed into soybean as described in Example 6.

#### EXAMPLE 12

##### Construction of Chimeric Genes for Expression of Corynebacterium DHDPs and SSP3-5 in the Embryo and Endosperm of Transformed Corn

The following chimeric genes were made for transformation into corn:

- globulin 1 promoter/mcts/cordapA/NOS 3 region
- glutelin 2 promoter/mcts/cordapA/NOS 3' region
- globulin 1 promoter/SSP3-5/globulin 1 3' region
- glutelin 2 promoter/SSP3-5/10 kD 3' region

The glutelin 2 promoter was cloned from corn genomic DNA using PCR with primers based on the published sequence [Reina et al. (1990) Nucleic Acids Res. 18:6426-6426]. The promoter fragment includes 1020 nucleotides upstream from the ATG translation start codon. An Nco I site was introduced via PCR at the ATG start site to allow for direct translational fusions. A BamH I site was introduced on the 5' end of the promoter. The 1.02 kb BamH I to Nco I promoter fragment was cloned into the BamH I to Nco I sites of the plant expression vector pML63 (see Example 11) replacing the 35S promoter to create vector pML90. This vector contains the glutelin 2 promoter linked to the GUS coding region and the NOS 3'.

The 10 kD zein 3' region was derived from a 10 kD zein gene clone generated by PCR from genomic DNA using oligonucleotide primers based on the published sequence [Kirihara et al. (1988) Gene 71:359-370]. The 3' region  
5 extends 940 nucleotides from the stop codon. Restriction endonuclease sites for Kpn I, Sma I and Xba I sites were added immediately following the TAG stop codon by oligonucleotide insertion to facilitate cloning. A Sma I to Hind III segment containing the  
10 10 kD 3' region was isolated and ligated into Sma I and Hind III digested pML90 to replace the NOS 3' sequence with the 10 kD 3' region, thus creating plasmid pML103. pML103 contains the glutelin 2 promoter, an Nco I site at the ATG start codon of the GUS gene, Sma I and Xba I  
15 sites after the stop codon, and 940 nucleotides of the 10 kD zein 3' sequence.

The globulin 1 promoter and 3' sequences were isolated from a Clontech corn genomic DNA library using oligonucleotide probes based on the published sequence  
20 of the globulin 1 gene [Kriz et al. (1989) Plant Physiol. 91:636]. The cloned segment includes the promoter fragment extending 1078 nucleotides upstream from the ATG translation start codon, the entire globulin coding sequence including introns and the 3'  
25 sequence extending 803 bases from the translational stop. To allow replacement of the globulin 1 coding sequence with other coding sequences an Nco I site was introduced at the ATG start codon, and Kpn I and Xba I sites were introduced following the translational stop  
30 codon via PCR to create vector pCC50. There is a second Nco I site within the globulin 1 promoter fragment. The globulin 1 gene cassette is flanked by Hind III sites.

The plant amino acid biosynthetic enzymes are known to be localized in the chloroplasts and therefore are  
35 synthesized with a chloroplast targeting signal.

Bacterial proteins such as DHDPS have no such signal. A chloroplast transit sequence (cts) was therefore fused to the ~~cordapa~~ coding sequence in the chimeric genes described below. For corn the cts used was based on the  
5 the cts of the small subunit of ribulose 1,5-bisphosphate carboxylase from corn [Lebrun et al. (1987) Nucleic Acids Res. 15:4360] and is designated acts to distinguish it from the soybean cts. The oligonucleotides SEQ ID NOS:93-98 were synthesized and used  
10 essentially as described in Example 4.

Oligonucleotides SEQ ID NO:93 and SEQ ID NO:94, which encode the carboxy terminal part of the corn chloroplast targeting signal, were annealed, resulting in Xba I and Nco I compatible ends, purified via  
15 polyacrylamide gel electrophoresis, and inserted into Xba I plus Nco I digested pBT492 (see Example 3). The insertion of the correct sequence was verified by DNA sequencing yielding pBT556. Oligonucleotides SEQ ID NO:95 and SEQ ID NO:96, which encode the middle part of  
20 the chloroplast targeting signal, were annealed, resulting in Bgl II and Xba I compatible ends, purified via polyacrylamide gel electrophoresis, and inserted into Bgl II and Xba I digested pBT556. The insertion of the correct sequence was verified by DNA sequencing  
25 yielding pBT557. Oligonucleotides SEQ ID NO:97 and SEQ ID NO:98, which encode the amino terminal part of the chloroplast targeting signal, were annealed, resulting in Nco I and Afl II compatible ends, purified via polyacrylamide gel electrophoresis, and inserted into  
30 Nco I and Afl II digested pBT557. The insertion of the correct sequence was verified by DNA sequencing yielding pBT558. Thus the mcts was fused to the lysC-M4 gene.

A DNA fragment containing the entire mcts was prepared using PCR. The template DNA was pBT558 and the  
35 oligonucleotide primers used were:

SEQ ID NO:99:

GCGCCCACCG TGATGA

5 SEQ ID NO:100:

CACCGGATTC TTCCGC

The mcts fragment was linked to the amino terminus of the DHDPS protein encoded by ecodapA gene by  
10 digesting with Nco I and treating with the Klenow fragment of DNA polymerase to fill in the 5' overhangs. The inserted fragment and the vector/insert junctions were determined to be correct by DNA sequencing, yielding pBT576.

15 To construct the chimeric gene:

globulin 1 promoter/mcts/cordapA/NOS 3' region  
an Nco I to Kpn I fragment containing the mcts/ecodapA  
coding sequence was isolated from plasmid pBT576 (see  
Example 6) and inserted into Nco I plus Kpn I digested  
20 pCC50 creating plasmid pBT662. Then the ecodapA coding  
sequence was replaced with the cordapA coding sequence  
as follows. An Afl II to Kpn I fragment containing the  
distal two thirds of the mcts fused to the cordapA  
coding sequence was inserted into Afl II to Kpn I  
25 digested pBT662 creating plasmid pBT677.

To construct the chimeric gene:

glutelin 2 promoter/mcts/cordapA/NOS 3' region  
an Nco I to Kpn I fragment containing the mcts/cordapA  
coding sequence was isolated from plasmid pBT677 and  
30 inserted into Nco I to Kpn I digested pML90, creating  
plasmid pBT679.

To construct the chimeric gene:

glutelin 2 promoter/SSP3-5/10 kD 3' region  
the plasmid pML103 (above) containing the glutelin 2  
35 promoter and 10 kD zein 3' region was cleaved at the  
Nco I and Sma I sites. The SSP3-5 coding region

(Example 9) was isolated as an Nco I to blunt end fragment by cleaving with Xba I followed by filling in the sticky end using Klenow fragment of DNA polymerase, then cleaving with Nco I. The 193 base pair Nco I to blunt end fragment was ligated into the Nco I and Sma I cut pML103 to create pLH104.

To construct the chimeric gene:  
globulin 1 promoter/SSP3-5/globulin 1 3' region  
the 193 base pair Nco I and Xba I fragment containing  
the SSP3-5 coding region (Example 9) was inserted into  
plasmid pCC50 (above) which had been cleaved with Xba I  
to completion and then partially cut with Nco I to open  
the plasmid at the ATG start codon creating pLH105.

#### EXAMPLE 13

#### Corn Plants Containing Chimeric Genes for Expression of Corynebacterium DHDPS in the Embryo and Endosperm

Corn was transformed with the chimeric genes:  
globulin 1 promoter/mcts/cordapa/NOS 3' region  
or  
glutelin 2 promoter/mcts/cordapa/NOS 3' region

Either one of two plasmid vectors containing  
selectable markers were used in the transformations.  
One plasmid, pDETRIC, contained the bar gene from  
Streptomyces hygrosopicus that confers resistance to  
the herbicide glufosinate [Thompson et al. (1987) The  
EMBO Journal 6:2519-2523]. The bacterial gene had its  
translation codon changed from GTG to ATG for proper  
translation initiation in plants [De Block et al. (1987)  
The EMBO Journal 6:2513-2518]. The bar gene was driven  
by the 35S promoter from Cauliflower Mosaic Virus and  
uses the termination and polyadenylation signal from the  
octopine synthase gene from Agrobacterium tumefaciens.  
Alternatively, the selectable marker used was 35S/Ac, a  
synthetic phosphinothricin-N-acetyltransferase (pat)

gene under the control of the 35S promoter and 3' terminator/polyadenylation signal from Cauliflower Mosaic Virus [Eckes et.al., (1989) J Cell Biochem Suppl 13 D].

5 Embryogenic callus cultures were initiated from immature embryos (about 1.0 to 1.5 mm) dissected from kernels of a corn line bred for giving a "type II callus" tissue culture response. The embryos were dissected 10 to 12 d after pollination and were placed  
10 with the axis-side down and in contact with agarose-solidified N6 medium [Chu et al., (1974) Sci Sin 18:659-668] supplemented with 0.5 mg/L 2,4-D (N6-0.5). The embryos were kept in the dark at 27°C. Friable embryogenic callus consisting of undifferentiated masses  
15 of cells with somatic proembryos and somatic embryos borne on suspensor structures proliferated from the scutellum of the immature embryos. Clonal embryogenic calli isolated from individual embryos were identified and sub-cultured on N6-0.5 medium every 2 to 3 weeks.

20 The particle bombardment method was used to transfer genes to the callus culture cells. A Biolistic, PDS-1000/He (BioRAD Laboratories, Hercules, CA) was used for these experiments.

Circular plasmid DNA or DNA which had been  
25 linearized by restriction endonuclease digestion was precipitated onto the surface of gold particles. DNA from two or three different plasmids, one containing the selectable marker for corn transformation, and one or two containing the chimeric genes for increased lysine  
30 accumulation in seeds were co-precipitated. To accomplish this 1.5 µg of each DNA (in water at a concentration of about 1 mg/mL) was added to 25 mL of gold particles (average diameter of 1.5 µm) suspended in water (60 mg of gold per mL). Calcium chloride (25 mL  
35 of a 2.5 M solution) and spermidine (10 mL of a 1.0 M



solution) were then added to the gold-DNA suspension as the tube was vortexing. The gold particles were centrifuged in a microfuge for 10 sec and the supernatant removed. The gold particles were then  
5 resuspended in 200 mL of absolute ethanol, were centrifuged again and the supernatant removed. Finally, the gold particles were resuspended in 25 mL of absolute ethanol and sonicated twice for one sec. Five  $\mu$ L of the DNA-coated gold particles were then loaded on each macro  
10 carrier disk and the ethanol was allowed to evaporate away leaving the DNA-covered gold particles dried onto the disk.

Embryogenic callus (from the callus line designated #LH132.5.X) was arranged in a circular area of about  
15 6 cm in diameter in the center of a 100 X 20 mm petri dish containing N6-0.5 medium supplemented with 0.25M sorbitol and 0.25M mannitol. The tissue was placed on this medium for 2 h prior to bombardment as a pretreatment and remained on the medium during the  
20 bombardment procedure. At the end of the 2 h pretreatment period, the petri dish containing the tissue was placed in the chamber of the PDS-1000/He. The air in the chamber was then evacuated to a vacuum of 28 inch of Hg. The macrocarrier was accelerated with a  
25 helium shock wave using a rupture membrane that bursts when the He pressure in the shock tube reaches 1100 psi. The tissue was placed approximately 8 cm from the stopping screen. Four plates of tissue were bombarded with the DNA-coated gold particles. Immediately  
30 following bombardment, the callus tissue was transferred to N6-0.5 medium without supplemental sorbitol or mannitol.

Within 24 h after bombardment the tissue was transferred to selective medium, N6-0.5 medium that  
35 contained 2 mg/L glufosinate and lacked casein or

proline. Tissue that continued to grow slowly on this medium was transferred to fresh N6-0.5 medium supplemented with glufosinate every 2 weeks. After 6-12 weeks clones of actively growing callus were identified. Callus was then transferred to medium that promotes plant regeneration.

Plants regenerated from transformed callus were analyzed for the presence of the intact transgenes via Southern blot or PCR. The plants were selfed or outcrossed to an elite line to generate R1 or F1 seeds, respectively. Single R1 seeds or six to eight F1 seeds were pooled and assayed for expression of the Corynebacterium DHGPS protein by western blot analysis. The free amino acid composition and total amino acid composition of the seeds were determined as described in previous examples.

Expression of the Corynebacterium DHGPS protein, driven by either the globulin or glutelin promoter, was observed in the corn seeds (Table 8). Free lysine levels in the seeds increased from about 1.4% of free amino acids in control seeds to 15-27% in seeds expressing Corynebacterium DHGPS from the globulin 1 promoter. The higher DHGPS expression and higher lysine level in the selfed seed probably results from the fact that half of the pooled seeds in the outcrossed lines are expected to lack the transgene due to segregation. A smaller increase in free lysine was observed in seeds expressing Corynebacterium DHGPS from the glutelin 2 promoter. Thus to increase lysine, it may be better to express this enzyme in the embryo rather than the endosperm. A high level of saccharopine, indicative of lysine catabolism, was observed in seeds the contained high levels of lysine.

Lysine normally represents about 2.3% of the seed amino acid content. It is therefore apparent from

Table 8 that substantial increases (35%-130%) in lysine as a percent of total seed amino acids was found in seeds expressing Corynebacterium DHDPS from the globulin 1 promoter.

5

TABLE 8

1088.1.2 line: globulin 1 promoter/mcts/cordapA/NOS 3 region  
 1099.2.1 line: globulin 1 promoter/mcts/cordapA/NOS 3 region  
 1090.2.1 line: glutelin 2 promoter/mcts/cordapA/NOS 3' region

TRANSGENIC LINE	WESTERN	% LYS of FREE SEED AMINO ACIDS	% LYS of TOTAL SEED AMINO ACIDS
	CORYNE. DHDPS		
1088.1.2 x elite	+	15	3.1
1099.2.1 selfed	++	27	5.3
1090.2.1 x elite	+	2.3	1.7

## EXAMPLE 14

Transformation of Soybean with the Kunitz trypsin inhibitor 3 promoter/cts/cordapA Chimeric Gene

10 A seed-specific expression cassette composed of the promoter and transcription terminator from the the soybean Kunitz trypsin inhibitor 3 (KTI3) gene [Jofuku et al. (1989) Plant Cell 1:427-435] was created. The KTI3 cassette includes about 2000 nucleotides upstream  
 15 (5') from the translation initiation codon and about 200 nucleotides downstream (3') from the translation stop codon of Kunitz trypsin inhibitor 3. Between the 5' and 3' regions restriction endonuclease sites Nco I (which includes the ATG translation initiation codon) and Kpn I  
 20 were created to permit insertion of the Corynebacterium dapA gene. The entire cassette was flanked by BamH I and Sal I sites.

As described in Example 4 a chloroplast transit sequence (cts) was fused to the dapA coding sequence in  
 25 the chimeric gene. The cts used was based on the the cts of the small subunit of ribulose 1,5-bisphosphate carboxylase from soybean [Berry-Lowe et al. (1982) J.

Mol. Appl. Genet. 1:483-498]. A 1030 bp Nco I-Kpn I fragment containing the cts attached to the cordapA coding region was isolated from an agarose gel following electrophoresis and inserted into the KTI3 expression  
5 cassette yielding plasmid pML102 (Figure 15).

Plasmid pML102 was introduced into soybean by particle-mediated bombardment by Agracetus Company (Middleton, WI), according to the procedure described in United States Patent No. 5,015,580. To screen for  
10 transformed cells, plasmid pML102 was co-bombarded with another plasmid carrying a soybean transformation marker gene consisting of the 35S promoter from Cauliflower Mosaic Virus driving expression of the *E. coli*  $\beta$ -glucuronidase (GUS) gene [Jefferson et al. (1986)  
15 Proc. Natl. Acad. Sci. USA 83:8447-8451] with the Nos 3' region.

It was expected that the transgenes would be segregating in the R1 seeds of the transformed plants. To identify seeds that carried the transformation marker  
20 gene, a small chip of the seed was cut off with a razor and put into a well in a disposable plastic microtiter plate. A GUS assay mix consisting of 100 mM  $\text{NaH}_2\text{PO}_4$ , 10 mM EDTA, 0.5 mM  $\text{K}_4\text{Fe}(\text{CN})_6$ , 0.1% Triton X-100, 0.5 mg/mL 5-Bromo-4-chloro-3-indolyl  $\beta$ -D-glucuronic acid  
25 was prepared and 0.15 mL was added to each microtiter well. The microtiter plate was incubated at 37° for 45 minutes. The development of blue color indicated the expression of GUS in the seed.

To measure the total amino acid composition of  
30 mature seeds, 1-1.4 milligrams of the seed meal was hydrolyzed in 6N hydrochloric acid, 0.4%  $\beta$ -mercapto-ethanol under nitrogen for 24 h at 110-120°C; 1/50 of the sample was run on a Beckman Model 6300 amino acid analyzer using post-column ninhydrin detection. Lysine  
35 (and other amino acid) levels in the seeds were compared

as percentages of the total amino acids. Wild type soybean seeds contain 5.7-6.0% lysine.

One hundred fifty individual seeds from sixteen independent transformed lines were analyzed (Table 9).

5 Ten of the sixteen lines had seeds with a lysine content of 7% of the total seed amino acids or greater, a 16-22% increase over wild type seeds. Thus, more than 62% of the transformation events had co-integrated the plasmid carrying the cordapA gene along with the plasmid bearing  
10 the marker GUS gene. About 80% of the high lysine seeds were GUS positive, suggesting that the plasmid carrying the cordapA gene usually integrated at the same chromosomal site as the plasmid carrying the GUS gene. However, in some transformed lines, e.g. 260-05, there  
15 was little correlation between the GUS positive and high lysine phenotypes, indicating that the two plasmids integrated at unlinked sites. Both of these types of transformation events were expected based upon the procedure used for this transformation.

20 Seeds with a lysine content greater than 20% of the total seed amino acids were obtained. This represents nearly a three hundred percent increase in seed lysine content.

TABLE 9

<u>LINE #</u>	<u>SEED #</u>	<u>GUS</u>	<u>%LYS</u>
257-1	G1	+	8.30
	G2	+	7.99
	G3	+	11.51
	G4	+	8.52
	G33	+	7.68
	G34	+	9.93
	G35	-	5.97
	G36	-	5.71
	G37	+	7.48

G38	+	9.42
G39	+	10.44
G40	+	8.63
G41	+	9.42
G42	+	8.53
G43	+	10.54
G44	-	5.83
G45	+	7.15
G46	+	7.85
G47	+	7.34

## 257-21

G21	+	12.90
G22	+	11.52
G23	+	9.34
G24	-	5.82
G25	-	5.61
G26	-	5.70
G27	-	5.84
G28	-	14.27
G48	+	15.23
G49	+	18.79
G50	+	13.82
G51	-	5.94
G52	+	13.29
G53	+	14.61

## 257-41

G54	+	6.28
G55	+	6.27
G56	+	6.32
G57	+	6.4
G180	+	5.75
G181	+	7.42

## 257-46

G60	+	6.76
G61	+	6.73
G62	-	6.18

105

	G63	+	6.13
	G182	+	6.83
	G183	+	6.23
257-49	G78	-	6.40
	G79	+	6.46
	G184	+	6.37
	G185	+	6.15
	G186	+	6.41
	G187	+	7.90
257-50	G88	-	6.15
	G89	-	6.12
	G188	+	6.19
	G189	+	6.07
	G190	+	6.09
	G191	+	6.30
257-51	G228	-	5.81
	G229	-	5.74
	G230	-	5.59
	G231	-	6.00
	G232	-	5.89
	G233	+	21.49
	G234	+	20.30
	G235	+	11.89
	G236	+	12.40
	G237	+	15.09
	G238	+	12.79
	G239	+	17.19
260-05	G90	-	5.41
	G91	-	7.65
	G95	-	6.39
	G96	-	5.80
	G97	-	6.12

106

	G98	-	5.90
	G99	-	6.17
	G160	-	8.04
	G161	-	12.64
	G162	-	6.91
	G163	-	5.83
	G164	-	8.28
	G165	-	12.52
	G166	-	5.68
	G167	-	9.92
	G168	-	5.89
	G169	-	6.10
	G170	+	6.49
	G171	+	6.10
	G172	-	12.83
	G173	-	6.55
	G174	-	6.62
	G175	+	13.02
	G176	-	10.13
	G177	-	5.97
	G178	-	11.37
	G179	-	12.63
260-13	G108	+	6.64
	G109	+	7.92
	G192	+	10.29
	G193	+	7.37
	G194	+	6.73
	G195	+	10.35
260-16	G29	+	11.64
	G30	+	14.87
	G31	+	15.02
	G32	-	6.24



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260-17	G115	+	11.91
	G116	-	6.21
	G117	-	6.08
	G118	-	6.28
	G119	-	6.30
	G196	+	7.76
260-23	G129	+	5.93
	G197	+	6.04
	G198	+	5.99
	G199	+	6.11
	G200	+	6.35
	G201	+	6.19
260-31	G202	+	6.19
	G203	+	6.19
	G204	+	6.13
	G205	+	6.40
	G206	+	6.73
	G207	+	6.23
260-33	G217	+	6.80
	G218	-	7.00
	G219	-	6.80
	G220	-	6.10
	G221	-	6.83
	G222	-	6.18
	G223	+	5.92
	G224	+	6.61
	G226	+	6.17
	G227	+	6.43
	G240	+	6.25
	G241	+	6.13
260-44	G148	+	6.51
	G149	+	6.21

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260-46

G208	+	6.02
G209	+	6.17
G210	+	6.12
G211	+	6.09
G158	-	6.00
G159	+	6.30
G212	+	6.40
G213	+	6.50
G214	+	6.40
G215	+	6.60

SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT:
  - (A) NAME: E. I. DU PONT DE NEMOURS AND COMPANY
  - (B) STREET: 1007 MARKET STREET
  - (C) CITY: WILMINGTON
  - (D) STATE: DELAWARE
  - (E) COUNTRY: UNITED STATES OF AMERICA
  - (F) POSTAL CODE (ZIP): 19898
  - (G) TELEPHONE: 302-992-4931
  - (H) TELEFAX: 302-773-0164
  - (I) TELEX: 6717325
- (ii) TITLE OF INVENTION: CHIMERIC GENES AND METHODS FOR INCREASING THE LYSINE CONTENT OF THE SEEDS OF CORN, SOYBEAN AND RAPESEED PLANTS
- (iii) NUMBER OF SEQUENCES: 100
- (iv) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: DISKETTE, 3.50 INCH
  - (B) COMPUTER: MACINTOSH
  - (C) OPERATING SYSTEM: MACINTOSH, 6.0
  - (D) SOFTWARE: MICROSOFT WORD, 4.0
- (v) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vi) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: 08/160,117
  - (B) FILING DATE: NOVEMBER 30, 1993
- (vii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: BARBARA C. SIEGELL
  - (B) REGISTRATION NUMBER: 30,684
  - (C) REFERENCE/DOCKET NUMBER: BB-1055-B

110

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 48 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCCGGGCCAT GGCTACAGGT TTAACAGCTA AGACCGGAGT AGAGCACT 48

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 37 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GATATCGAAT TCTCATTATA GAACTCCAGC TTTTTC 37

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 917 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 3..911

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CC ATG GCT ACA GGT TTA ACA GCT AAG ACC GGA GTA GAG CAC TTC GGC 47  
 Met Ala Thr Gly Leu Thr Ala Lys Thr Gly Val Glu His Phe Gly  
     1                    5                    10                    15

ACC GTT GGA GTA GCA ATG GTT ACT CCA TTC ACG GAA TCC GGA GAC ATC 95  
 Thr Val Gly Val Ala Met Val Thr Pro Phe Thr Glu Ser Gly Asp Ile  
                     20                    25                    30

111

GAT ATC GCT GCT GGC CGC GAA GTC GCG GCT TAT TTG GTT GAT AAG GGC 143  
 Asp Ile Ala Ala Gly Arg Glu Val Ala Ala Tyr Leu Val Asp Lys Gly  
 35 40 45

TTG GAT TCT TTG GTT CTC GCG GGC ACC ACT GGT GAA TCC CCA ACG ACA 191  
 Leu Asp Ser Leu Val Leu Ala Gly Thr Thr Gly Glu Ser Pro Thr Thr  
 50 55 60

ACC GCC GCT GAA AAA CTA GAA CTG CTC AAG GCC GTT CGT GAG GAA GTT 239  
 Thr Ala Ala Glu Lys Leu Glu Leu Leu Lys Ala Val Arg Glu Glu Val  
 65 70 75

GGG GAT CGG GCG AAG CTC ATC GCC GGT GTC GGA ACC AAC AAC ACG CGG 287  
 Gly Asp Arg Ala Lys Leu Ile Ala Gly Val Gly Thr Asn Asn Thr Arg  
 80 85 90 95

ACA TCT GTG GAA CTT GCG GAA GCT GCT GCT TCT GCT GGC GCA GAC GGC 335  
 Thr Ser Val Glu Leu Ala Glu Ala Ala Ser Ala Gly Ala Asp Gly  
 100 105 110

CTT TTA GTT GTA ACT CCT TAT TAC TCC AAG CCG AGC CAA GAG GGA TTG 383  
 Leu Leu Val Val Thr Pro Tyr Tyr Ser Lys Pro Ser Gln Glu Gly Leu  
 115 120 125

CTG GCG CAC TTC GGT GCA ATT GCT GCA GCA ACA GAG GTT CCA ATT TGT 431  
 Leu Ala His Phe Gly Ala Ile Ala Ala Thr Glu Val Pro Ile Cys  
 130 135 140

CTC TAT GAC ATT CCT GGT CGG TCA GGT ATT CCA ATT GAG TCT GAT ACC 479  
 Leu Tyr Asp Ile Pro Gly Arg Ser Gly Ile Pro Ile Glu Ser Asp Thr  
 145 150 155

ATG AGA CGC CTG AGT GAA TTA CCT ACG ATT TTG GCG GTC AAG GAC GCC 527  
 Met Arg Arg Leu Ser Glu Leu Pro Thr Ile Leu Ala Val Lys Asp Ala  
 160 165 170 175

AAG GGT GAC CTC GTT GCA GCC ACG TCA TTG ATC AAA GAA ACG GGA CTT 575  
 Lys Gly Asp Leu Val Ala Ala Thr Ser Leu Ile Lys Glu Thr Gly Leu  
 180 185 190

GCC TGG TAT TCA GGC GAT GAC CCA CTA AAC CTT GTT TGG CTT GCT TTG 623  
 Ala Trp Tyr Ser Gly Asp Asp Pro Leu Asn Leu Val Trp Leu Ala Leu  
 195 200 205

GGC GGA TCA GGT TTC ATT TCC GTA ATT GGA CAT GCA GCC CCC ACA GCA 671  
 Gly Gly Ser Gly Phe Ile Ser Val Ile Gly His Ala Ala Pro Thr Ala  
 210 215 220

TTA CGT GAG TTG TAC ACA AGC TTC GAG GAA GGC GAC CTC GTC CGT GCG 719  
 Leu Arg Glu Leu Tyr Thr Ser Phe Glu Glu Gly Asp Leu Val Arg Ala  
 225 230 235

CGG GAA ATC AAC GCC AAA CTA TCA CCG CTG GTA GCT GCC CAA GGT CGC 767  
 Arg Glu Ile Asn Ala Lys Leu Ser Pro Leu Val Ala Ala Gln Gly Arg  
 240 245 250 255

112

CTT GAG GCT CTC CGA GAA GAC ATG AAA AAA GCT GGA GTT CTA TAA TGAGAAATTC 918  
Leu Glu Ala Leu Arg Glu Asp Met Lys Lys Ala Gly Val Leu \*  
290 295 300

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTTCCCGTGA CCATGGGCCA TC

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1350 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..1350

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATG GCT GAA ATT GTT GTC TCC AAA TTT GGC GGT ACC AGC GTA GCT GAT 48  
Met Ala Glu Ile Val Val Ser Lys Phe Gly Gly Thr Ser Val Ala Asp  
1 5 10 15

TTT GAC GCC ATG AAC CGC AGC GCT GAT ATT GTG CTT TCT GAT GCC AAC 96  
Phe Asp Ala Met Asn Arg Ser Ala Asp Ile Val Leu Ser Asp Ala Asn  
20 25 30

GTG CGT TTA GTT GTC CTC TCG GCT TCT GCT GGT ATC ACT AAT CTG CTG 144  
Val Arg Leu Val Val Leu Ser Ala Ser Ala Gly Ile Thr Asn Leu Leu  
35 40 45

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GTC GCT TTA GCT GAA GGA CTG GAA CCT GGC GAG CGA TTC GAA AAA CTC	192
Val Ala Leu Ala Glu Gly Leu Glu Pro Gly Glu Arg Phe Glu Lys Leu	
50 55 60	
GAC GCT ATC CGC AAC ATC CAG TTT GCC ATT CTG GAA CGT CTG CGT TAC	240
Asp Ala Ile Arg Asn Ile Gln Phe Ala Ile Leu Glu Arg Leu Arg Tyr	
65 70 75 80	
CCG AAC GTT ATC CGT GAA GAG ATT GAA CGT CTG CTG GAG AAC ATT ACT	288
Pro Asn Val Ile Arg Glu Glu Ile Glu Arg Leu Leu Glu Asn Ile Thr	
85 90 95	
GTT CTG GCA GAA GCG GCG GCG CTG GCA ACG TCT CCG GCG CTG ACA GAT	336
Val Leu Ala Glu Ala Ala Leu Ala Thr Ser Pro Ala Leu Thr Asp	
100 105 110	
GAG CTG GTC AGC CAC GGC GAG CTG ATG TCG ACC CTG CTG TTT GTT GAG	384
Glu Leu Val Ser His Gly Glu Leu Met Ser Thr Leu Leu Phe Val Glu	
115 120 125	
ATC CTG CGC GAA CGC GAT GTT CAG GCA CAG TGG TTT GAT GTA CGT AAA	432
Ile Leu Arg Glu Arg Asp Val Gln Ala Gln Trp Phe Asp Val Arg Lys	
130 135 140	
GTG ATG CGT ACC AAC GAC CGA TTT GGT CGT GCA GAG CCA GAT ATA GCC	480
Val Met Arg Thr Asn Asp Arg Phe Gly Arg Ala Glu Pro Asp Ile Ala	
145 150 155 160	
GCG CTG GCG GAA CTG GCC GCG CTG CAG CTG CTC CCA CGT CTC AAT GAA	528
Ala Leu Ala Glu Leu Ala Ala Leu Gln Leu Leu Pro Arg Leu Asn Glu	
165 170 175	
GGC TTA GTG ATC ACC CAG GGA TTT ATC GGT AGC GAA AAT AAA GGT CGT	576
Gly Leu Val Ile Thr Gln Gly Phe Ile Gly Ser Glu Asn Lys Gly Arg	
180 185 190	
ACA ACG ACG CTT GGC CGT GGA GGC AGC GAT TAT ACG GCA GCC TTG CTG	624
Thr Thr Thr Leu Gly Arg Gly Gly Ser Asp Tyr Thr Ala Ala Leu Leu	
195 200 205	
GCG GAG GCT TTA CAC GCA TCT CGT GTT GAT ATC TGG ACC GAC GTC CCG	672
Ala Glu Ala Leu His Ala Ser Arg Val Asp Ile Trp Thr Asp Val Pro	
210 215 220	
GGC ATC TAC ACC ACC GAT CCA CGC GTA GTT TCC GCA GCA AAA CGC ATT	720
Gly Ile Tyr Thr Thr Asp Pro Arg Val Val Ser Ala Ala Lys Arg Ile	
225 230 235 240	
GAT GAA ATC GCG TTT GCC GAA GCG GCA GAG ATG GCA ACT TTT GGT GCA	768
Asp Glu Ile Ala Phe Ala Glu Ala Ala Glu Met Ala Thr Phe Gly Ala	
245 250 255	
AAA GTA CTG CAT CCG GCA ACG TTG CTA CCC GCA GTA CGC AGC GAT ATC	816
Lys Val Leu His Pro Ala Thr Leu Leu Pro Ala Val Arg Ser Asp Ile	
260 265 270	

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CCG	GTC	TTT	GTC	GGC	TCC	AGC	AAA	GAC	CCA	CGC	GCA	GGT	GGT	ACG	CTG	864
Pro	Val	Phe	Val	Gly	Ser	Ser	Lys	Asp	Pro	Arg	Ala	Gly	Gly	Thr	Leu	
		275					280					285				
GTG	TGC	AAT	AAA	ACT	GAA	AAT	CCG	CCG	CTG	TTC	CGC	GCT	CTG	GCG	CTT	912
Val	Cys	Asn	Lys	Thr	Glu	Asn	Pro	Pro	Leu	Phe	Arg	Ala	Leu	Ala	Leu	
	290					295					300					
CGT	CGC	AAT	CAG	ACT	CTG	CTC	ACT	TTG	CAC	AGC	CTG	AAT	ATG	CTG	CAT	960
Arg	Arg	Asn	Gln	Thr	Leu	Leu	Thr	Leu	His	Ser	Leu	Asn	Met	Leu	His	
305					310					315					320	
TCT	CGC	GGT	TTC	CTC	GCG	GAA	GTT	TTC	GGC	ATC	CTC	GCG	CGG	CAT	AAT	1008
Ser	Arg	Gly	Phe	Leu	Ala	Glu	Val	Phe	Gly	Ile	Leu	Ala	Arg	His	Asn	
				325					330					335		
ATT	TCG	GTA	GAC	TTA	ATC	ACC	ACG	TCA	GAA	GTG	AGC	GTG	GCA	TTA	ACC	1056
Ile	Ser	Val	Asp	Leu	Ile	Thr	Thr	Ser	Glu	Val	Ser	Val	Ala	Leu	Thr	
			340					345					350			
CTT	GAT	ACC	ACC	GGT	TCA	ACC	TCC	ACT	GGC	GAT	ACG	TTG	CTG	ACG	CAA	1104
Leu	Asp	Thr	Thr	Gly	Ser	Thr	Ser	Thr	Gly	Asp	Thr	Leu	Leu	Thr	Gln	
		355					360					365				
TCT	CTG	CTG	ATG	GAG	CTT	TCC	GCA	CTG	TGT	CGG	GTG	GAG	GTG	GAA	GAA	1152
Ser	Leu	Leu	Met	Glu	Leu	Ser	Ala	Leu	Cys	Arg	Val	Glu	Val	Glu	Glu	
	370					375					380					
GGT	CTG	GCG	CTG	GTC	GCG	TTG	ATT	GGC	AAT	GAC	CTG	TCA	AAA	GCC	TGC	1200
Gly	Leu	Ala	Leu	Val	Ala	Leu	Ile	Gly	Asn	Asp	Leu	Ser	Lys	Ala	Cys	
385					390					395				400		
GCC	GTT	GGC	AAA	GAG	GTA	TTC	GGC	GTA	CTG	GAA	CCG	TTC	AAC	ATT	CGC	1248
Ala	Val	Gly	Lys	Glu	Val	Phe	Gly	Val	Leu	Glu	Pro	Phe	Asn	Ile	Arg	
			405					410						415		
ATG	ATT	TGT	TAT	GGC	GCA	TCC	AGC	CAT	AAC	CTG	TGC	TTC	CTG	GTG	CCC	1296
Met	Ile	Cys	Tyr	Gly	Ala	Ser	Ser	His	Asn	Leu	Cys	Phe	Leu	Val	Pro	
			420					425					430			
GGC	GAA	GAT	GCC	GAG	CAG	GTG	GTG	CAA	AAA	CTG	CAT	AGT	AAT	TTG	TTT	1344
Gly	Glu	Asp	Ala	Glu	Gln	Val	Val	Gln	Lys	Leu	His	Ser	Asn	Leu	Phe	
		435					440					445				
GAG	TAA															1350
Glu	*															
	450															

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)



- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:  
GATCCATGGC TGAAATTGTT GTCTCCAAAT TTGGCG 36
- (2) INFORMATION FOR SEQ ID NO:7:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:  
GTACCGCCAA ATTTGGAGAC AACAAATTCA GCCATG 36
- (2) INFORMATION FOR SEQ ID NO:8:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 75 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:  
CATGGCTGGC TTCCCCACGA GGAAGACCAA CAATGACATT ACCTCCATTG CTAGCAACGG 60  
TGGAAGAGTA CAATG 75
- (2) INFORMATION FOR SEQ ID NO:9:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 75 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:  
CATGCATTGT ACTCTTCCAC CGTTGCTAGC AATGGAGGTA ATGTCATTGT TGGTCTTCCT 60  
CGTGGGGAAG CCAGC 75

116

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 90 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CATGGCTTCC TCAATGATCT CCTCCCCAGC TGTTACCACC GTCAACCGTG CCGGTGCCGG 60  
CATGGTTGCT CCATTCACCG GCCTCAAAAG 90

## (2) INFORMATION FOR SEQ ID NO:11:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 90 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CATGCTTTTG AGGCCGGTGA ATGGAGCAAC CATGCCGGCA CCGGCACGGT TGACGGTGGT 60  
AACAGCTGGG GAGGAGATCA TTGAGGAAGC 90

## (2) INFORMATION FOR SEQ ID NO:12:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCGGTTTGCT GTAATAGGTA CCA 23

## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

117

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AGCTTGGTAC CTATTACAGC AAACCGGCAT G

31

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCTTCCTCAA TGATCTCCTC CCCAGCT

27

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CATTGTACTC TTCCACCGTT GCTAGCAA

28

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: misc\_feature

(B) LOCATION: 1..20

(D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
70"

118

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CTGACTCGCT GCGCTCGGTC

20

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..24
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
71"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TATTTTCTCC TTACGCATCT GTGC

24

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..27
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
78"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TTCATCGATA GGCGACCACA CCCGTCC

27

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid

119

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..27
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
79"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AATATCGATG CCACGATGCG TCCGGCG

27

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 55 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..55
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
81"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CATGGAGGAG AAGATGAAGG CGATGGAAGA GAAGATGAAG GCGTGATAGG TACCG

55

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 55 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..55

120

(D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
80"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AATTCGGTAC CTATCACGCC TTCATCTTCT CTTCCATCGC CTTTCATCTTC TCCTC 55

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: unknown  
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

(A) NAME/KEY: Protein  
(B) LOCATION: 1..14  
(D) OTHER INFORMATION: /label= name  
/note= "base gene  
[(SSP5)2]"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Ala  
1 5 10

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: misc\_feature  
(B) LOCATION: 1..21  
(D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
84"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GATGGAGGAG AAGATGAAGG C

21

121

## (2) INFORMATION FOR SEQ ID NO:24:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..21
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
85"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ATCGCCTTCA TCTTCTCCTC C

21

## (2) INFORMATION FOR SEQ ID NO:25:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..21
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
82"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GATGGAGGAG AAGCTGAAGG C

21

## (2) INFORMATION FOR SEQ ID NO:26:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..21
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
83"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ATCGCCTTCA GCTTCTCCTC C

21

## (2) INFORMATION FOR SEQ ID NO:27:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Met Glu Glu Lys Leu Lys Ala  
1 5

## (2) INFORMATION FOR SEQ ID NO:28:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Met Glu Glu Lys Met Lys Ala  
1 5

## (2) INFORMATION FOR SEQ ID NO:29:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 160 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)



123

- (vi) ORIGINAL SOURCE:  
 (B) STRAIN: E. coli  
 (G) CELL TYPE: DH5 alpha
- (vii) IMMEDIATE SOURCE:  
 (B) CLONE: C15
- (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 2..151  
 (D) OTHER INFORMATION: /function= "synthetic  
 storage protein"  
 /product= "protein"  
 /gene= "ssp"  
 /standard\_name=  
 "5.7.7.7.7.5"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

```

C ATG GAG GAG AAG ATG AAG GCG ATG GAG GAG AAG CTG AAG GCG ATG   46
  Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Leu Lys Ala Met
    1             5             10             15

GAG GAG AAG CTG AAG GCG ATG GAG GAG AAG CTG AAG GCG ATG GAG GAG   94
  Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu
                20             25             30

AAG CTG AAG GCG ATG GAG GAG AAG CTG AAG GCG ATG GAA GAG AAG ATG  142
  Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Met
                35             40             45

AAG GCG TGATAGGTAC CG                                           160
  Lys Ala
                50

```

## (2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 49 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

```

Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu
  1             5             10             15

Glu Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys
    20             25             30

```

124

Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Met Lys  
 35 40 45

Ala

## (2) INFORMATION FOR SEQ ID NO:31:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 160 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (vi) ORIGINAL SOURCE:

- (B) STRAIN: E. coli
- (G) CELL TYPE: DH5 alpha

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: C20

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 2..151
- (D) OTHER INFORMATION: /function="synthetic storage protein"  
 /product="protein"  
 /gene="ssp"  
 /standard name="5.7.7.7.7.5"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

C ATG GAG GAG AAG ATG AAG GCG ATG GAG GAG AAG CTG AAG GCG ATG 46  
 Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Leu Lys Ala Met  
 1 5 10 15

GAG GAG AAG CTG AAG GCG ATG GAG GAG AAG CTG AAG GCG ATG GAG GAG 94  
 Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu  
 20 25 30

AAG CTG AAG GCG ATG GAG GAG AAG CTG AAG GCG ATG GAA GAG AAG ATG 142  
 Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Met  
 35 40 45

AAG GCG TGATAGGTAC CG 160  
 Lys Ala  
 50

## (2) INFORMATION FOR SEQ ID NO:32:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 amino acids

125

(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

```

Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu
 1             5             10             15
Glu Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys
      20             25             30
Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Met Lys
      35             40             45

```

Ala

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 139 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(B) STRAIN: E. coli  
(G) CELL TYPE: DH5 alpha

(vii) IMMEDIATE SOURCE:

(B) CLONE: C30

(ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: 2..130  
(D) OTHER INFORMATION: /function= "synthetic  
storage protein"  
/product= "protein"  
/gene= "ssp"  
/standard\_name=  
"5.7.7.7.5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

```

C ATG GAG GAG AAG ATG AAG GCG ATG GAG GAG AAG CTG AAG GCG ATG      46
  Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Leu Lys Ala Met
    1             5             10             15
GAG GAG AAG CTG AAG GCG ATG GAG GAG AAG CTG AAG GCG ATG GAG GAG      94
  Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu
    20             25             30

```

AAG CTG AAG GCG ATG GAA GAG AAG ATG AAG GCG TGATAGGTAC CG 139  
Lys Leu Lys Ala Met Glu Glu Lys Met Lys Ala  
35 40

## (2) INFORMATION FOR SEQ ID NO:34:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu  
1 5 10 15  
Glu Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys  
20 25 30  
Leu Lys Ala Met Glu Glu Lys Met Lys Ala  
35 40

## (2) INFORMATION FOR SEQ ID NO:35:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 97 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (vi) ORIGINAL SOURCE:

- (B) STRAIN: E. coli
- (G) CELL TYPE: DH5 alpha

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: D16

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 2..88
- (D) OTHER INFORMATION: /function= "synthetic  
storage protein"  
/product= "protein"  
/gene= "ssp"  
/standard\_name=  
"5.5.5.5"

127

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

C ATG GAG GAG AAG ATG AAG GCG ATG GAG GAG AAG ATG AAG GCG ATG 46  
 Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Ala Met  
     1                    5                    10                    15

GAG GAG AAG ATG AAG GCG ATG GAA GAG AAG ATG AAG GCG TGATAGGTAC 95  
 Glu Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Ala  
                     20                    25

CG

97

## (2) INFORMATION FOR SEQ ID NO:36:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Ala Met Glu  
     1                    5                    10                    15

Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Ala  
                     20                    25

## (2) INFORMATION FOR SEQ ID NO:37:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 118 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (vi) ORIGINAL SOURCE:

- (B) STRAIN: E. coli
- (G) CELL TYPE: DH5 alpha

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: D20

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 2..109
- (D) OTHER INFORMATION: /function= "synthetic  
 storage protein"  
 /product= "protein"  
 /gene= "ssp"

/standard\_name=  
"5.5.5.5.5"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

```

C ATG GAG GAG AAG ATG AAG GCG ATG GAG GAG AAG ATG AAG GCG ATG   46
  Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Ala Met
    1             5             10             15

GAG GAG AAG ATG AAG GCG ATG GAG GAG AAG ATG AAG GCG ATG GAA GAG   94
  Glu Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Ala Met Glu Glu
                20             25             30

AAG ATG AAG GCG TGATAGGTAC CG                                     118
  Lys Met Lys Ala
                35

```

## (2) INFORMATION FOR SEQ ID NO:38:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 35 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

```

Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Ala Met Glu
  1             5             10             15

Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys
    20             25             30

Met Lys Ala
    35

```

## (2) INFORMATION FOR SEQ ID NO:39:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 97 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:  
 (B) STRAIN: E. coli  
 (G) CELL TYPE: DH5 alpha

(vii) IMMEDIATE SOURCE:  
 (B) CLONE: D33

129

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 2..88
- (D) OTHER INFORMATION: /function= "synthetic storage protein"  
/product= "protein"  
/gene= "ssp"  
/standard\_name= "5.5.5.5"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

C ATG GAG GAG AAG ATG AAG GCG ATG GAG GAG AAG ATG AAG GCG ATG 46  
Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Ala Met  
1 5 10 15

GAG GAG AAG ATG AAG GCG ATG GAA GAG AAG ATG AAG GCG TGATAGGTAC 95  
Glu Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Ala  
20 25

CG 97

## (2) INFORMATION FOR SEQ ID NO:40:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Ala Met Glu  
1 5 10 15

Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Ala  
20 25

## (2) INFORMATION FOR SEQ ID NO:41:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..21
- (D) OTHER INFORMATION: /product= "synthetic oligonucleotide"

130

/standard\_name= "SM  
86"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

GATGGAGGAG AAGCTGAAGA A

21

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..21
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
87"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

ATCTTCTTCA GCTTCTCCTC C

21

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..21
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
88"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GATGGAGGAG AAGCTGAAGT G

21



131

## (2) INFORMATION FOR SEQ ID NO:44:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..21
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
89"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

ATCCACTTCA GCTTCTCCTC C

21

## (2) INFORMATION FOR SEQ ID NO:45:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..21
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
90"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

GATGGAGGAG AAGATGAAGA A

21

## (2) INFORMATION FOR SEQ ID NO:46:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..21
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
91"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

ATCTTCTTCA TCTTCTCCTC C

21

## (2) INFORMATION FOR SEQ ID NO:47:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..21
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
92"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

GATGGAGGAG AAGATGAAGT G

21

## (2) INFORMATION FOR SEQ ID NO:48:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..21
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
93"

133

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

ATCCAATTCA TCTTCTCCTC C

21

(2) INFORMATION FOR SEQ ID NO:49:

- (i) SEQUENCE CHARACTERISTICS: -  
(A) LENGTH: 7 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: unknown  
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

Met Glu Glu Lys Leu Lys Lys  
1 5

(2) INFORMATION FOR SEQ ID NO:50:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: unknown  
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

Met Glu Glu Lys Leu Lys Trp  
1 5

(2) INFORMATION FOR SEQ ID NO:51:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: unknown  
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

Met Glu Glu Lys Met Lys Lys  
1 5

(2) INFORMATION FOR SEQ ID NO:52:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7 amino acids

134

(B) TYPE: amino acid  
 (C) STRANDEDNESS: unknown  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

Met Glu Glu Lys Met Lys Trp  
 1 5

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 160 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(B) STRAIN: E. coli  
 (G) CELL TYPE: DH5 alpha

(vii) IMMEDIATE SOURCE:

(B) CLONE: 82-4

(ix) FEATURE:

(A) NAME/KEY: CDS  
 (B) LOCATION: 2..151  
 (D) OTHER INFORMATION: /function="synthetic  
 storage protein  
 /product="protein"  
 /gene="ssp"  
 /standard\_name=  
 "7.7.7.7.7.5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

C ATG GAG GAG AAG CTG AAG GCG ATG GAG GAG AAG CTG AAG GCG ATG 46  
 Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met  
 1 5 10 15

GAG GAG AAG CTG AAG GCG ATG GAG GAG AAG CTG AAG GCG ATG GAG GAG 94  
 Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu  
 20 25 30

AAG CTG AAG GCG ATG GAG GAG AAG CTG AAG GCG ATG GAA GAG AAG ATG 142  
 Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Met  
 35 40 45

135

AAG GCG TGATAGGTAC CG  
Lys Ala

160

50

## (2) INFORMATION FOR SEQ ID NO:54:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

```

Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu
 1               5               10               15
Glu Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys
      20               25               30
Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Met Lys
 35               40               45
Ala

```

## (2) INFORMATION FOR SEQ ID NO:55:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 97 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (vi) ORIGINAL SOURCE:

- (B) STRAIN: E. coli
- (G) CELL TYPE: DH5 alpha

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: 84-H3

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 2..88
- (D) OTHER INFORMATION: /function= "synthetic  
storage protein  
/product= "protein"  
/gene= "ssp"  
/standard\_name=  
"5.5.5.5"

136

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

C ATG GAG GAG AAG ATG AAG GCG ATG GAG GAG AAG ATG AAG GCG ATG 46  
Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Ala Met  
1 5 10 15

GAG GAG AAG ATG AAG GCG ATG GAA GAG AAG ATG AAG GCG TGATAGGTAC 95  
Glu Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Ala  
20 25

CG 97

## (2) INFORMATION FOR SEQ ID NO:56:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Ala Met Glu  
1 5 10 15

Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Ala  
20 25

## (2) INFORMATION FOR SEQ ID NO:57:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 97 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (vi) ORIGINAL SOURCE:

- (B) STRAIN: E. coli
- (G) CELL TYPE: DH5 alpha

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: 86-H23

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 2..88
- (D) OTHER INFORMATION: /function= "synthetic  
storage protein  
/product= "protein"  
/gene= "ssp"

137

/standard\_name=  
"5.8.8.5"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

C ATG GAG GAG AAG ATG AAG GCG ATG GAG GAG AAG CTG AAG AAG ATG 46  
Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Leu Lys Lys Met  
1 5 10 15

GAG GAG AAG CTG AAG AAG ATG GAA GAG AAG ATG AAG GCG TGATAGGTAC 95  
Glu Glu Lys Leu Lys Lys Met Glu Glu Lys Met Lys Ala  
20 25

CG

97

## (2) INFORMATION FOR SEQ ID NO:58:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Leu Lys Lys Met Glu  
1 5 10 15

Glu Lys Leu Lys Lys Met Glu Glu Lys Met Lys Ala  
20 25

## (2) INFORMATION FOR SEQ ID NO:59:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 112 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (vi) ORIGINAL SOURCE:

- (B) STRAIN: E. coli
- (G) CELL TYPE: DH5 alpha

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: 88-2

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 2..103
- (D) OTHER INFORMATION: /function= "synthetic storage protein"

138

/product= "protein"  
/gene= "ssp"  
/standard\_name=  
"5.9.9.9.5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

C ATG GAG GAG AAG ATG AAG GCG AAG AAG CTG AAG TGG ATG GAG GAG .46  
Met Glu Glu Lys Met Lys Ala Lys Lys Leu Lys Trp Met Glu Glu  
1 5 10 15  
AAG CTG AAG TGG ATG GAG GAG AAG CTG AAG TGG ATG GAA GAG AAG ATG 94  
Lys Leu Lys Trp Met Glu Glu Lys Leu Lys Trp Met Glu Glu Lys Met  
20 25 30  
AAG GCG TGATAGGTAC CG 112  
Lys Ala

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

Met Glu Glu Lys Met Lys Ala Lys Lys Leu Lys Trp Met Glu Glu Lys  
1 5 10 15  
Leu Lys Trp Met Glu Glu Lys Leu Lys Trp Met Glu Glu Lys Met Lys  
20 25 30  
Ala

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 118 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (B) STRAIN: E. coli
- (G) CELL TYPE: DH5 alpha

(vii) IMMEDIATE SOURCE:

- (B) CLONE: 90-H8



139

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 2..109
- (D) OTHER INFORMATION: /function= "synthetic  
storage protein  
/product= "protein"  
/gene= "ssp"  
/standard name=  
"5.10.10.10.5"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

```

C ATG GAG GAG AAG ATG AAG GCG ATG GAG GAG AAG ATG AAG AAG ATG 46
  Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Lys Met
    1             5             10             15

GAG GAG AAG ATG AAG AAG ATG GAG GAG AAG ATG AAG AAG ATG GAA GAG 94
  Glu Glu Lys Met Lys Lys Met Glu Glu Lys Met Lys Lys Met Glu Glu
                20             25             30

AAG ATG AAG GCG TGATAGGTAC CG 118
  Lys Met Lys Ala
                35

```

## (2) INFORMATION FOR SEQ ID NO:62:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

```

Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Lys Met Glu
  1             5             10             15

Glu Lys Met Lys Lys Met Glu Glu Lys Met Lys Lys Met Glu Glu Lys
                20             25             30

Met Lys Ala
                35

```

## (2) INFORMATION FOR SEQ ID NO:63:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 97 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

140

- (vi) ORIGINAL SOURCE:  
(B) STRAIN: E. coli  
(G) CELL TYPE: DH5 alpha
- (vii) IMMEDIATE SOURCE:  
(B) CLONE: 92-2
- (ix) FEATURE:  
(A) NAME/KEY: CDS  
(B) LOCATION: 2..88  
(D) OTHER INFORMATION: /function= "synthetic  
storage protein  
/product= "protein"  
/gene= "ssp"  
/standard name=  
"5.11.11.5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

C ATG GAG GAG AAG ATG AAG GCG ATG GAG GAG AAG ATG AAG TGG ATG 46  
Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Trp Met  
1 5 10 15

GAG GAG AAG ATG AAG TGG ATG GAA GAG AAG ATG AAG GCG TGATAGGTAC 95  
Glu Glu Lys Met Lys Trp Met Glu Glu Lys Met Lys Ala  
20 25

CG 97

(2) INFORMATION FOR SEQ ID NO:64:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 28 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Trp Met Glu  
1 5 10 15

Glu Lys Met Lys Trp Met Glu Glu Lys Met Lys Ala  
20 25

(2) INFORMATION FOR SEQ ID NO:65:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 84 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

141

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..84
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
96"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

GATGGAGGAA AAGATGAAGG CGATGGAGGA GAAAATGAAA GCTATGGAGG AAAAGATGAA 60  
AGCGATGGAG GAGAAAATGA AGGC 84

(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 84 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..84
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
97"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

ATCGCCTTCA TTTTCTCCTC CATCGCTTTC ATCTTTTCCT CCATAGCTTT CATTTTCTCC 60  
TCCATCGCCT TCATCTTTTC CTCC 84

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

- (A) NAME/KEY: Protein
- (B) LOCATION: 1..28

142

(D) OTHER INFORMATION: /label= name  
/note= "(SSP 5)4"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Ala Met Glu  
1 5 10 15  
Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Ala  
20 25

(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 84 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..84
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
98"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

GATGGAGGAA AAGCTGAAAG CGATGGAGGA GAAACTCAAG GCTATGGAAG AAAAGCTTAA 60  
AGCGATGGAG GAGAAACTGA AGGC 84

(2) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 84 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..84
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
99"

143

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

ATCGCCTTCA GTTCTCCTC CTACGCTTTA AGCTTTTCTT CCATAGCCTT GAGTTTCTCC 60  
TCCATCGCTT TCAGCTTTTC CTCC

84

## (2) INFORMATION FOR SEQ ID NO:70:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: protein

## (ix) FEATURE:

- (A) NAME/KEY: Protein
- (B) LOCATION: 1..28
- (D) OTHER INFORMATION: /label= name  
/note= "(SSP 7)4"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu  
1 5 10 15  
Glu Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala  
20 25

## (2) INFORMATION FOR SEQ ID NO:71:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 84 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..84
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
100"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

GATGGAGGAA AAGCTTAAGA AGATGGAAGA AAAGCTGAAA TGGATGGAGG AGAAACTCAA 60  
AAAGATGGAG GAAAAGCTTA AATG

84

144

## (2) INFORMATION FOR SEQ ID NO:72:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 84 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..84
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
101"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

ATCCATTTAA GCTTTTCCTC CTACTTTTGT AGTTTCTCCT CCATCCATT CAGCTTTTCT 60  
TCCATCTTCT TAAGCTTTTC CTCC 84

## (2) INFORMATION FOR SEQ ID NO:73:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

Met Glu Glu Lys Leu Lys Lys Met Glu Glu Lys Leu Lys Trp Met Glu  
1 5 10 15  
Glu Lys Leu Lys Lys Met Glu Glu Lys Leu Lys Trp  
20 25

## (2) INFORMATION FOR SEQ ID NO:74:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 243 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (vi) ORIGINAL SOURCE:

- (B) STRAIN: E. coli

145

(G) CELL TYPE: DH5 alpha

(vii) IMMEDIATE SOURCE:

(B) CLONE: 2-9

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 2..235

(D) OTHER INFORMATION: /function= "synthetic  
storage protein  
/product= "protein"  
/gene= "ssp"  
/standard name=  
"7.7.7.7.7.7.8.9.8.9.5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

```

C ATG GAG GAG AAG CTG AAG GCG ATG GAG GAG AAG CTG AAG GCG ATG      46
  Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met
    1             5             10             15

GAG GAG AAG CTG AAG GCG ATG GAG GAG AAG CTG AAG GCG ATG GAG GAG      94
Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu
                20             25             30

AAG CTG AAG GCG ATG GAG GAG AAG CTG AAG GCG ATG GAG GAA AAG CTT     142
Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Leu
                35             40             45

AAG AAG ATG GAA GAA AAG CTG AAA TGG ATG GAG GAG AAA CTC AAA AAG     190
Lys Lys Met Glu Glu Lys Leu Lys Trp Met Glu Glu Lys Leu Lys Lys
                50             55             60

ATG GAG GAA AAG CTT AAA TGG ATG GAA GAG AAG ATG AAG GCG TGATAGGTAC 242
Met Glu Glu Lys Leu Lys Trp Met Glu Glu Lys Met Lys Ala
    65             70             75

C                                                                243

```

(2) INFORMATION FOR SEQ ID NO:75:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 77 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

```

Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu
  1             5             10             15

Glu Lys L u Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys
    20             25             30

```

Leu	Lys	Ala	Met	Glu	Glu	Lys	Leu	Lys	Ala	Met	Glu	Glu	Lys	Leu	Lys
		35					40					45			
Lys	Met	Glu	Glu	Lys	Leu	Lys	Trp	Met	Glu	Glu	Lys	Leu	Lys	Lys	Met
	50					55					60				
Glu	Glu	Lys	Leu	Lys	Trp	Met	Glu	Glu	Lys	Met	Lys	Ala			
65					70					75					

(A) LENGTH: 175 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

(B) STRAIN: E. coli  
(G) CELL TYPE: DH5 alpha

(B) CLONE: 5-1

(D) OTHER INFORMATION: /function= "synthetic  
storage protein  
/product= "protein"  
/gene= "ssp"  
/standard\_name=  
"5.5.5.7.7.7.5"

C	ATG	GAG	GAG	AAG	ATG	AAG	GCG	ATG	GAG	GAG	AAG	ATG	AAG	GCG	ATG	46
Met	Glu	Glu	Lys	Met	Lys	Ala	Met	Glu	Glu	Lys	Met	Lys	Ala	Met		
1				5					10					15		
GAG	GAG	AAG	ATG	AAG	GCG	ATG	GAG	GAA	AAG	CTG	AAA	GCG	ATG	GAG	GAG	94
Glu	Glu	Lys	Met	Lys	Ala	Met	Glu	Glu	Lys	Leu	Lys	Ala	Met	Glu	Glu	
				20					25					30		
AAA	CTC	AAG	GCT	ATG	GAA	GAA	AAG	CTT	AAA	GCG	ATG	GAG	GAG	AAA	CTG	142
Lys	Leu	Lys	Ala	Met	Glu	Glu	Lys	Leu	Lys	Ala	Met	Glu	Glu	Lys	Leu	
			35					40						45		
AAG	GCC	ATG	GAA	GAG	AAG	ATG	AAG	GCG	TGATAG							179
Lys	Ala	Met	Glu	Glu	Lys	Met	Lys	Ala								
			50				55									



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## (2) INFORMATION FOR SEQ ID NO:77:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 56 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

```

Met Glu Glu Lys Met Lys Ala Met Glu Glu Lys Met Lys Ala Met Glu
 1             5             10             15
Glu Lys Met Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys
      20             25             30
Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Leu Lys
      35             40             45
Ala Met Glu Glu Lys Met Lys Ala
 50             55

```

## (2) INFORMATION FOR SEQ ID NO:78:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 187 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (vi) ORIGINAL SOURCE:

- (B) STRAIN: E. coli
- (G) CELL TYPE: DH5 alpha

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 3..173
- (D) OTHER INFORMATION: /function= "synthetic  
storage protein  
/product= "protein"  
/gene= "ssp"  
/standard\_name=  
"SSP-3-5"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

```

CC ATG GAG GAG AAG CTG AAG GCG ATG GAG GAG AAG CTG AAG GCG ATG
Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met
 1             5             10             15

```

148

```

GAG GAG AAG CTG AAG GCG ATG GAG GAG AAG CTG AAG GCG ATG GAG GAG 95
Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu
                20                25                30

AAG CTG AAG GCG ATG GAG GAG AAG CTG AAG GCG ATG GAG GAA AAG ATG 143
Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Met
                35                40                45

AAG GCG ATG GAA GAG AAG ATG AAG GCG TGATAGGTAC CGAATTC 187
Lys Ala Met Glu Glu Lys Met Lys Ala
                50                55

```

## (2) INFORMATION FOR SEQ ID NO:79:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 56 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

```

Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu
 1                5                10                15

Glu Lys Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys
 20                25                30

Leu Lys Ala Met Glu Glu Lys Leu Lys Ala Met Glu Glu Lys Met Lys
 35                40                45

Ala Met Glu Glu Lys Met Lys Ala
 50                55

```

## (2) INFORMATION FOR SEQ ID NO:80:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 61 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..61
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
107"

149

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

CATGGAGGAG AAGATGAAAA AGCTCGAAGA GAAGATGAAG GTCATGAAGT GATAGGTACC 60  
G 61

(2) INFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 61 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..61
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
106"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

AATTCGGTAC CTATCACTTC ATGACCTTCA TCTTCTCTTC GAGCTTTTTC ATCTTCTCCT 60  
C 61

(2) INFORMATION FOR SEQ ID NO:82:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

- (A) NAME/KEY: Protein
- (B) LOCATION: 1..16
- (D) OTHER INFORMATION: /label= name  
/note= "pSK34 base  
gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

Met Glu Glu Lys Met Lys Lys Leu Glu Glu Lys Met Lys Val Met Lys  
1 5 10 15

150

## (2) INFORMATION FOR SEQ ID NO:83:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 63 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..63
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
110"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

GCTGGAAGAA AAGATGAAGG CTATGGAGGA CAAGATGAAA TGGCTTGAGG AAAAGATGAA 60  
GAA 63

## (2) INFORMATION FOR SEQ ID NO:84:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 63 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..63
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
111"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

AGCTTCTTCA TCTTTTCCTC AAGCCATTTT ATCTTGTCCT CCATAGCCTT CATCTTTTCT 60  
TCC 63

## (2) INFORMATION FOR SEQ ID NO:85:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

151

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

```

Met Glu Glu Lys Met Lys Lys Leu Glu Glu Lys Met Lys Ala Met Glu
 1             5             10             15
Asp Lys Met Lys Trp Leu Glu Glu Lys Met Lys Lys Leu Glu Glu Lys
      20             25             30
Met Lys Val Met Lys
      35

```

(2) INFORMATION FOR SEQ ID NO:86:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 37 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

```

Met Glu Glu Lys Met Lys Lys Leu Glu Glu Lys Met Lys Ala Met Glu
 1             5             10             15
Asp Lys Met Lys Trp Leu Glu Glu Lys Met Lys Lys Leu Glu Glu Lys
      20             25             30
Met Lys Val Met Lys
      35

```

(2) INFORMATION FOR SEQ ID NO:87:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 62 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..62
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
112"

152

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

GCTCGAAGAA AGATGAAGGC AATGGAAGAC AAAATGAAGT GGCTTGAGGA GAAAATGAAG 60

AA

62

## (2) INFORMATION FOR SEQ ID NO:88:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 62 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..62
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
113"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

AGCTTCTTCA TTTTCTCCTC AAGCCACTTC ATTTTGTCTT CCATTGCCTT CATCTTTCTT 60

CG

62

## (2) INFORMATION FOR SEQ ID NO:89:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

Met Glu Glu Lys Met Lys Lys Leu Lys Glu Glu Met Ala Lys Met Lys  
1 5 10 15Asp Glu Met Trp Lys Leu Lys Glu Glu Met Lys Lys Leu Glu Glu Lys  
20 25 30Met Lys Val Met Lys  
35

## (2) INFORMATION FOR SEQ ID NO:90:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 63 base pairs

153

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..63
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
114"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

GCTCAAGGAG GAAATGGCTA AGATGAAAGA CGAAATCTGG AAAGTAAAG AGGAAATGAA 60  
GAA

63

(2) INFORMATION FOR SEQ ID NO:91:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 63 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..63
- (D) OTHER INFORMATION: /product= "synthetic  
oligonucleotide"  
/standard\_name= "SM  
115"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

AGCTTCTTCA TTTCCTCTTT CAGTTTCCAC ATTTCGTCTT TCATCTTAGC CATTTCCTCC 60  
TTG

63

(2) INFORMATION FOR SEQ ID NO:92:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 107 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

154

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

Met Glu Glu Lys Met Lys Lys Leu Lys Glu Glu Met Ala Lys Met Lys  
 1 5 10 15  
 Asp Glu Met Trp Lys Leu Lys Glu Glu Met Lys Lys Leu Glu Glu Lys  
 20 25 30  
 Met Lys Val Met Glu Glu Lys Met Lys Lys Leu Glu Glu Lys Met Lys  
 35 40 45  
 Ala Met Glu Asp Lys Met Lys Trp Leu Glu Glu Lys Met Lys Lys Leu  
 50 55 60  
 Glu Glu Lys Met Lys Val Met Glu Glu Lys Met Lys Lys Leu Glu Glu  
 65 70 75 80  
 Lys Met Lys Ala Met Glu Asp Lys Met Lys Trp Leu Glu Glu Lys Met  
 85 90 95  
 Lys Lys Leu Glu Glu Lys Met Lys Val Met Lys  
 100 105

## (2) INFORMATION FOR SEQ ID NO:93:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

CTAGAAGCCT CGGCAACGTC AGCAACGGCG GAAGAATCCG GTG 43

## (2) INFORMATION FOR SEQ ID NO:94:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

CATGCACCGG ATTCTTCGCG CGTTGCTGAC GTTGCCGAGG CTT 43

## (2) INFORMATION FOR SEQ ID NO:95:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 55 base pairs



155

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

GATCCCATGG CGCCCCTTAA GTCCACCGCC AGCCTCCCCG TCGCCCGCCG CTCCT 55

(2) INFORMATION FOR SEQ ID NO:96:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 55 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

CTAGAGGAGC GCGGGGCGAC GGGGAGGCTG GCGGTGGACT TAAGGGGCGC CATGG 55

(2) INFORMATION FOR SEQ ID NO:97:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 59 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:

CATGGCGCCC ACCGTGATGA TGGCCTCGTC GGCCACCGCC GTCGCTCCGT TCCAGGGGC 59

(2) INFORMATION FOR SEQ ID NO:98:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 59 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:

TTAAGCCCCT GGAACGGAGC GACGGCGGTG GCCGACGAGG CCATCATCAC GGTGGGCGC 59

156

## (2) INFORMATION FOR SEQ ID NO:99:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 16 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:99:

GCGCCCACCG TGATGA

16

## (2) INFORMATION FOR SEQ ID NO:100:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 16 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:100:

CACCGGATTC TTCCGC

16

CLAIMS

What is claimed is:

1. A chimeric gene wherein a nucleic acid  
fragment encoding dihydrodipicolinic acid synthase which  
5 is insensitive to inhibition by lysine is operably  
linked to a plant chloroplast transit sequence and to a  
plant seed-specific regulatory sequence.

2. The chimeric gene of Claim 1, wherein the  
nucleic acid fragment encoding dihydrodipicolinic acid  
10 synthase comprises the nucleotide sequence shown in SEQ  
ID NO:3: encoding dihydrodipicolinic acid synthase from  
Corynebacterium glutamicum and wherein the plant  
chloroplast transit sequence is derived from a gene  
encoding the small subunit of ribulose 1,5-bisphosphate  
15 carboxylase from Glycine max, and the seed-specific  
regulatory sequence is from the gene encoding the  $\beta$   
subunit of the seed storage protein phaseolin from the  
bean Phaseolus vulgaris or the seed-specific regulatory  
sequence is from the Kunitz trypsin inhibitor 3 gene  
20 from Glycine max.

3. A plant comprising in its genome the chimeric  
gene of Claim 1 or Claim 2.

4. Seed obtained from the plant of Claim 3.

5. A method for obtaining a plant wherein the  
25 seeds of the plant accumulate lysine at a level from ten  
percent to four hundred percent higher than do seeds of  
an untransformed plant comprising:

(a) transforming plant cells with the  
chimeric gene of Claim 1;

30 (b) regenerating fertile mature plants from  
the transformed plant cells obtained from step (a) under  
conditions suitable to obtain seeds;

(c) screening the progeny seed of step (b)  
for lysine content; and

(d) selecting those lines whose seeds contain increased levels of lysine.

6. A method for obtaining a dicot plant wherein the seeds of the plant accumulate lysine at a level from  
5 ten percent to one hundred percent higher than do seeds of an untransformed plant comprising:

(a) transforming dicot cells with the chimeric gene of Claim 1 or Claim 2;

(b) regenerating fertile mature plants from  
10 the transformed plant cells obtained from step (a) under conditions suitable to obtain seeds;

(c) screening the progeny seed of step (b) for lysine content; and

(d) selecting those lines whose seeds contain  
15 increased levels of lysine.

7. A method for obtaining a rapeseed plant wherein the seeds of the plant accumulate lysine at a level from ten percent to one hundred percent higher than do seeds of an untransformed plant comprising:

20 (a) transforming rapeseed cells with the chimeric gene of Claim 1 or Claim 2;

(b) regenerating fertile mature plants from the transformed plant cells obtained from step (a) under conditions suitable to obtain seeds;

25 (c) screening the progeny seed of step (b) for lysine content; and

(d) selecting those lines whose seeds contain increased levels of lysine.

8. A method for obtaining a soybean plant wherein  
30 the seeds of the plant accumulate lysine at a level from ten percent to four hundred percent higher than do seeds of an untransformed plant comprising:

(a) transforming soybean cells with the chimeric gene of Claim 1 or Claim 2;

(b) regenerating fertile mature plants from the transformed plant cells obtained from step (a) under conditions suitable to obtain seeds;

(c) screening the progeny seed of step (b) for lysine content; and

(d) selecting those lines whose seeds contain increased levels of lysine.

9. A transformed plant wherein the seeds of the plant accumulate lysine at a level at least ten percent higher than do seeds of an untransformed plant.

10. A transformed plant, as described by Claim 9, wherein the seeds of the plant accumulate lysine at a level from ten percent to four hundred percent higher than do seeds of an untransformed plant.

11. A transformed rapeseed plant wherein the seeds of the plant accumulate lysine to a level between ten percent and one hundred percent higher than do seeds of an untransformed plant.

12. A transformed soybean plant wherein the seeds of the plant accumulate lysine to a level between ten percent and four hundred percent higher than do seeds of an untransformed plant.

13. A chimeric gene of Claim 1 wherein the seed-specific regulatory sequence is a monocot embryo-specific promoter.

14. The chimeric gene of Claim 1, wherein the nucleic acid fragment encoding dihydrodipicolinic acid synthase comprises the nucleotide sequence shown in SEQ ID NO:3: encoding dihydrodipicolinic acid synthase from Corynebacterium glutamicum and wherein the plant chloroplast transit sequence is derived from a gene encoding the small subunit of ribulose 1,5-bisphosphate carboxylase from Zea maize, and the seed-specific regulatory sequence is from the globulin 1 gene from Zea maize.

15. A method for obtaining a monocot plant wherein the seeds of the plant accumulate lysine at a level from ten percent to one hundred thirty percent higher than do seeds of an untransformed plant comprising:

- 5 (a) transforming monocot cells with the chimeric gene of Claim 13 or 14;
- (b) regenerating fertile mature plants from the transformed plant cells obtained from step (a) under conditions suitable to obtain seeds;
- 10 (c) screening the progeny seed of step (b) for lysine content; and
- (d) selecting those lines whose seeds contain increased levels of lysine.

16. A method for obtaining a corn plant wherein  
15 the seeds of the plant accumulate lysine at a level from ten percent to one hundred thirty percent higher than do seeds of an untransformed plant comprising:

- (a) transforming corn cells with the chimeric gene of Claim 13 or 14;
- 20 (b) regenerating fertile mature plants from the transformed plant cells obtained from step (a) under conditions suitable to obtain seeds;
- (c) screening the progeny seed of step (b) for lysine content; and
- 25 (d) selecting those lines whose seeds contain increased levels of lysine.

17. A monocot plant comprising in its genome the chimeric gene of Claim 13 or 14.

18. Seeds obtained from the plant of Claim 17.

30 19. A transformed monocot plant wherein the seeds of the plant accumulate lysine to a level between thirty-five percent and one hundred thirty percent higher than do seeds of an untransformed plant.

20. A transformed monocot plant of Claim 19  
35 wherein the plant is corn.

21. A nucleic acid fragment comprising:

(a) a first chimeric gene of Claim 1, 2, 13  
or 14 and

5 (b) a second chimeric gene wherein a nucleic acid fragment encoding a lysine-rich protein, wherein the weight percent lysine is at least 15%, is operably linked to a plant seed-specific regulatory sequence.

22. A nucleic acid fragment comprising:

10 (a) a first chimeric gene of Claim 1, 2, 13 or 14 and

(b) a second chimeric gene wherein a nucleic acid fragment encoding a lysine-rich protein comprises a nucleic acid sequence encoding a protein comprising n heptad units (d e f g a b c), each heptad being either  
15 the same or different, wherein:

n is at least 4;

a and d are independently selected from the group consisting of Met, Leu, Val, Ile and Thr;

20 e and g are independently selected from the group consisting of the acid/base pairs Glu/Lys, Lys/Glu, Arg/Glu, Arg/Asp, Lys/Asp, Glu/Arg, Asp/Arg and Asp/Lys; and

25 b, c and f are independently any amino acids except Gly or Pro and at least two amino acids of b, c and f in each heptad are selected from the group consisting of Glu, Lys, Asp, Arg, His, Thr, Ser, Asn, Ala, Gln and Cys,

30 said nucleic acid fragment is operably linked to a plant seed-specific regulatory sequence.

23. A nucleic acid fragment comprising:

35 (a) a first chimeric gene of Claim 1, 2, 13 or 14 and

(b) a second chimeric gene wherein a nucleic acid fragment encoding a lysine-rich protein comprises a nucleic acid sequence encoding a protein having the amino acid sequence (MEEKLKA)<sub>6</sub>(MEEKMKA)<sub>2</sub> is operably  
5 linked to a plant seed-specific regulatory sequence.

24. A plant comprising in its genome the chimeric gene of Claim 1, 2, 13 or 14 and the second chimeric gene of Claim 21, Claim 22 or Claim 23.

25. A plant comprising in its genome the nucleic  
10 acid fragment of Claim 21, Claim 22 or Claim 23.

26. Seed obtained from the plant of Claim 24.

27. Seed obtained from the plant of Claim 25.

28. A nucleic acid fragment comprising

(a) a first chimeric gene of Claim 1, 2, 13 or  
15 14 and

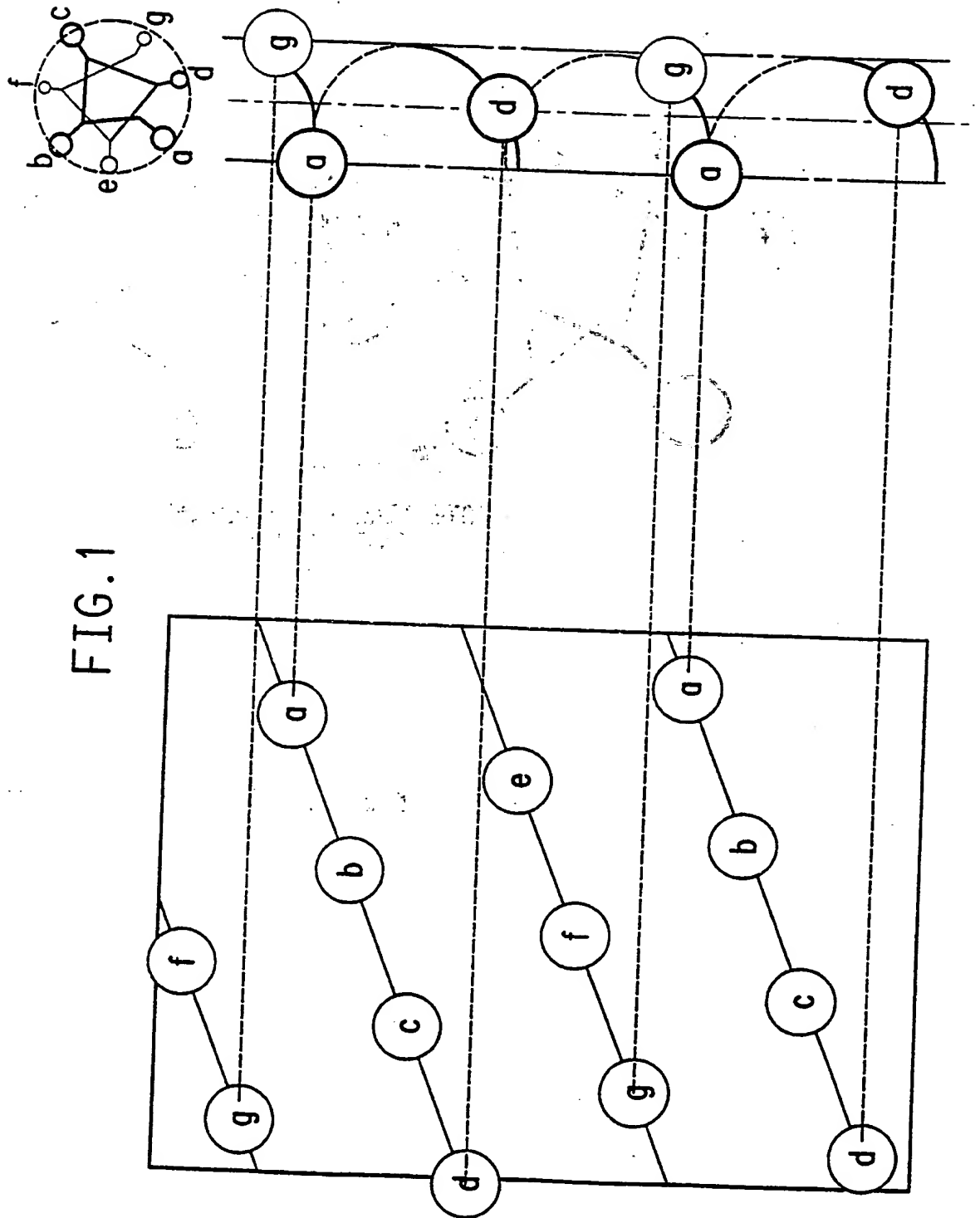
(b) a second chimeric gene wherein a nucleic acid fragment encoding a lysine ketoglutarate reductase is operably linked in the sense or antisense orientation to a plant seed-specific regulatory sequence.

20 29. A plant comprising in its genome the first chimeric gene of Claim 1, 2, 13 or 14 and a second chimeric gene wherein a nucleic acid fragment encoding a lysine ketoglutarate reductase is operably linked in the sense or antisense orientation to a plant seed-specific  
25 regulatory sequence.

30. A plant comprising in its genome the nucleic acid fragment of Claim 28.

31. Seed obtained from the plant of Claim 30.





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FIG. 2A

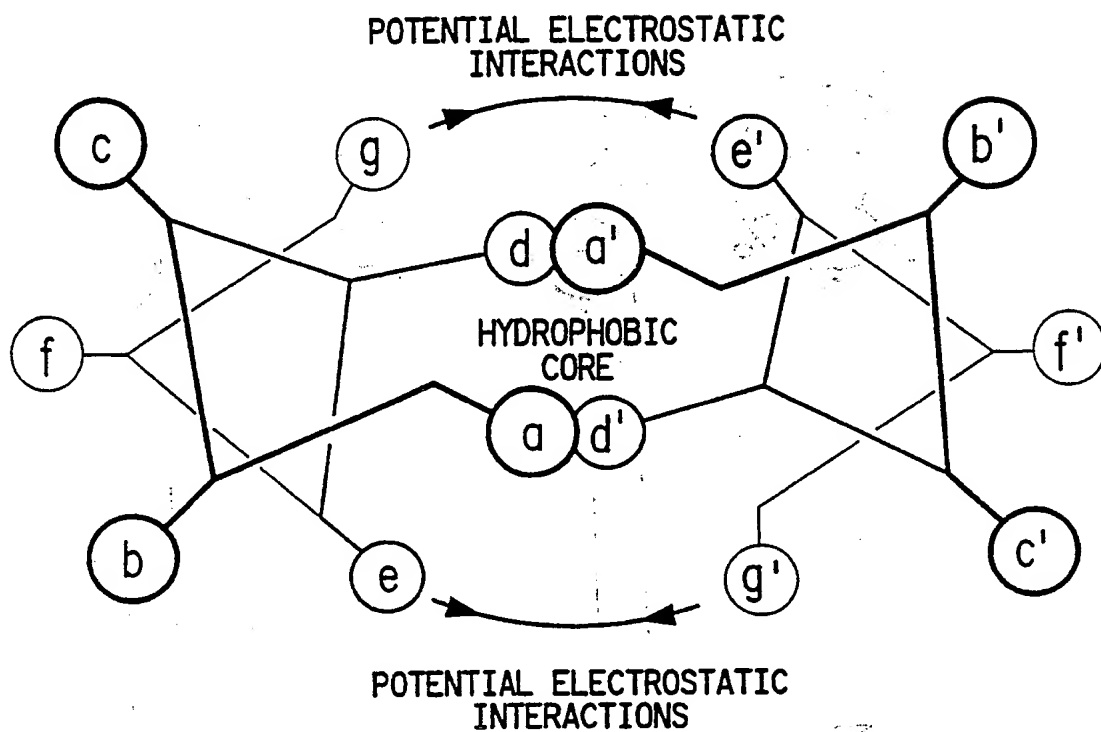


FIG. 2B

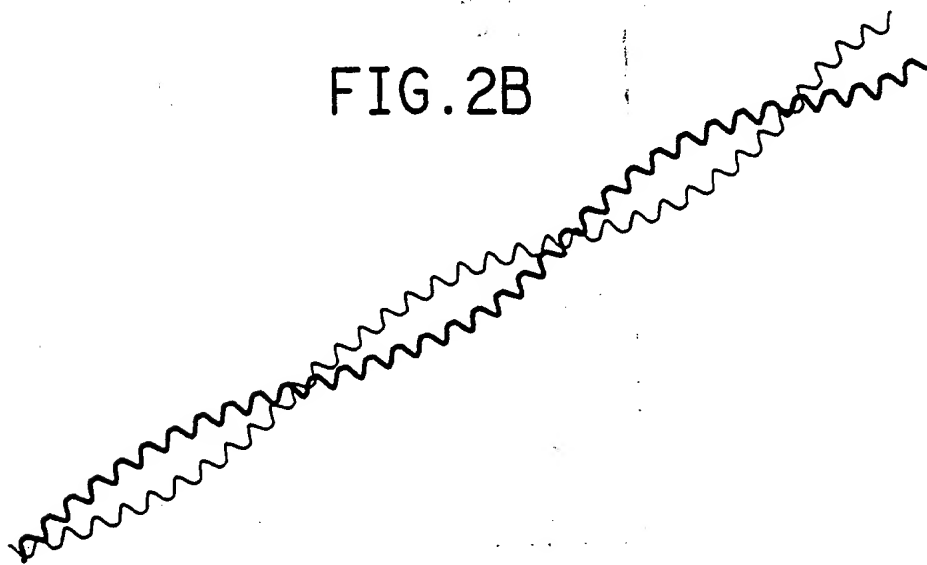


FIG. 3A

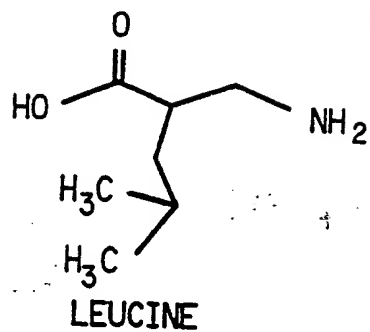


FIG. 3B

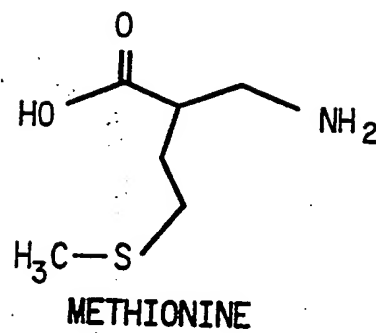
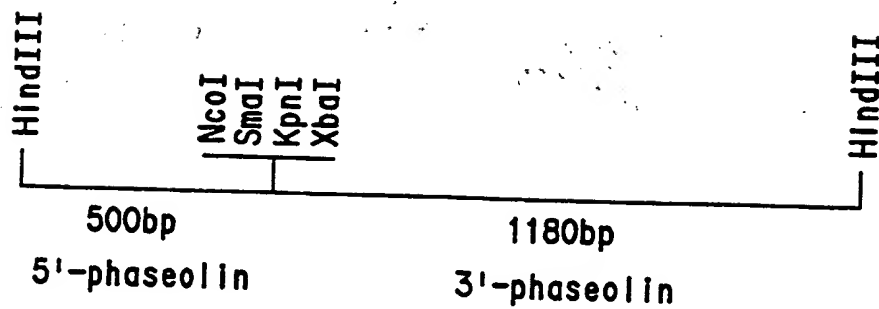


FIG. 4



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FIG. 5A

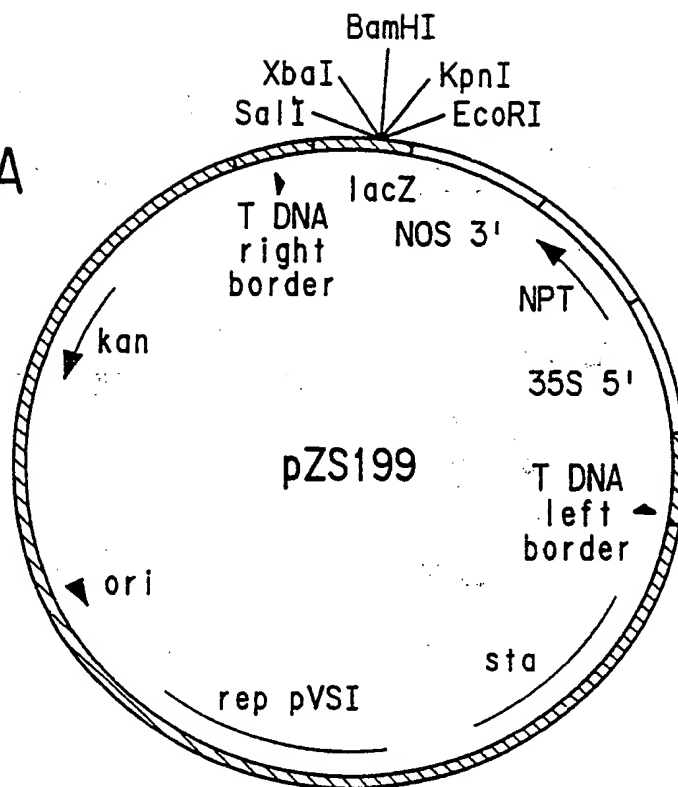
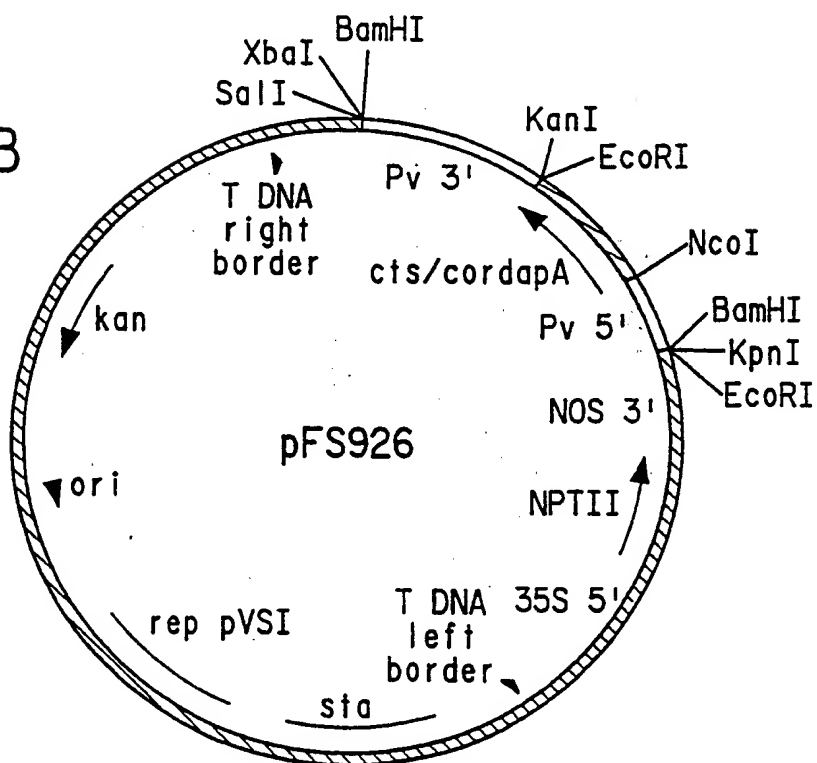


FIG. 5B



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FIG. 6A

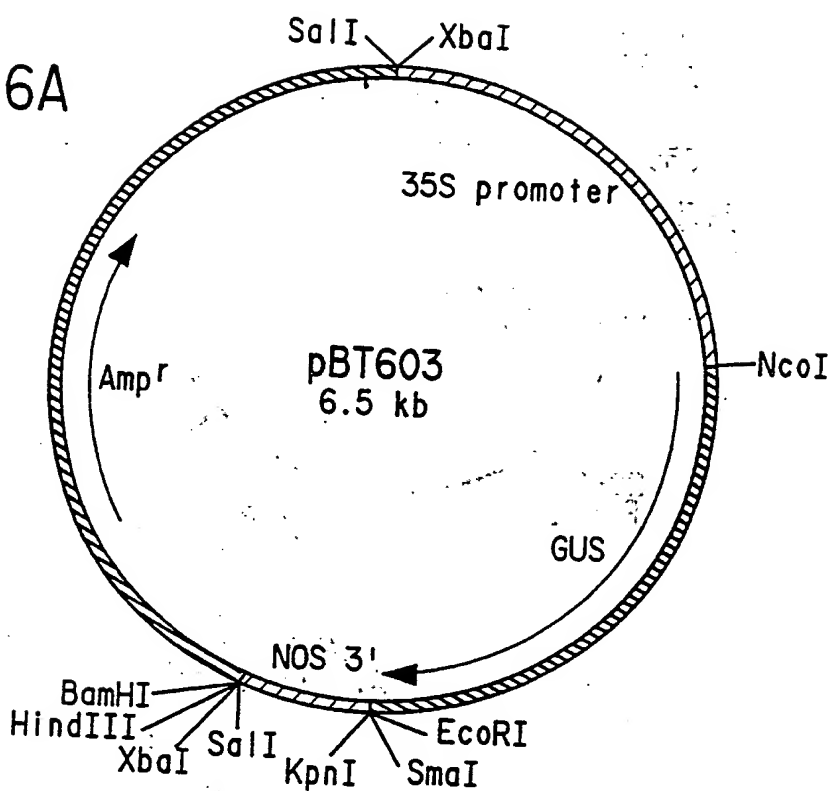
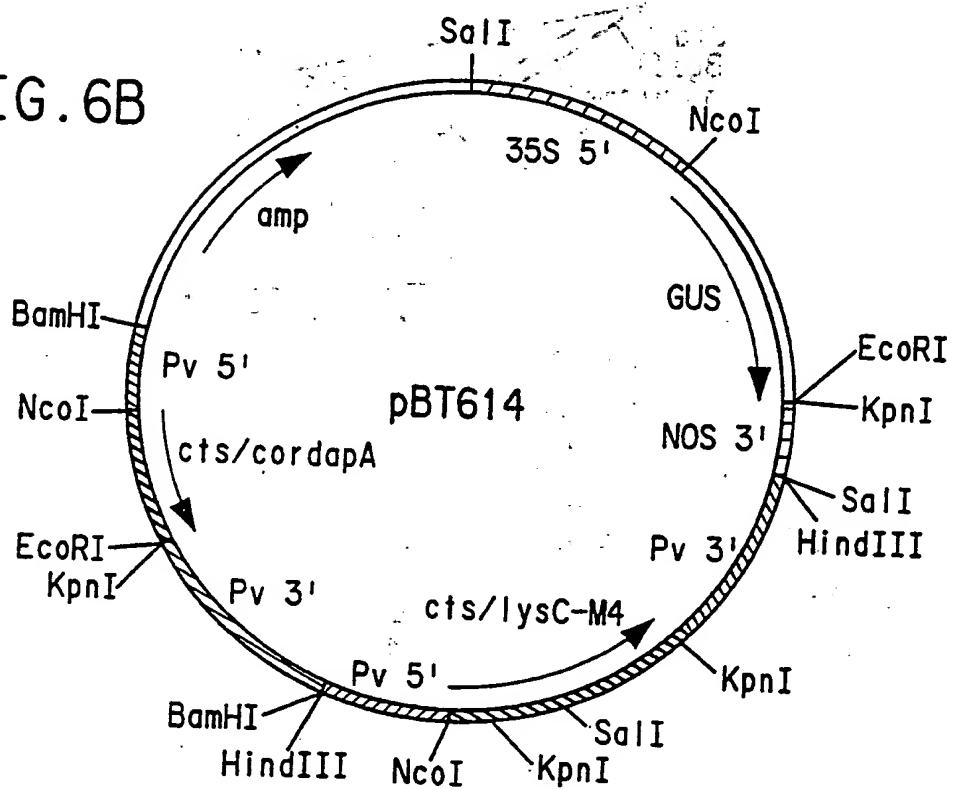
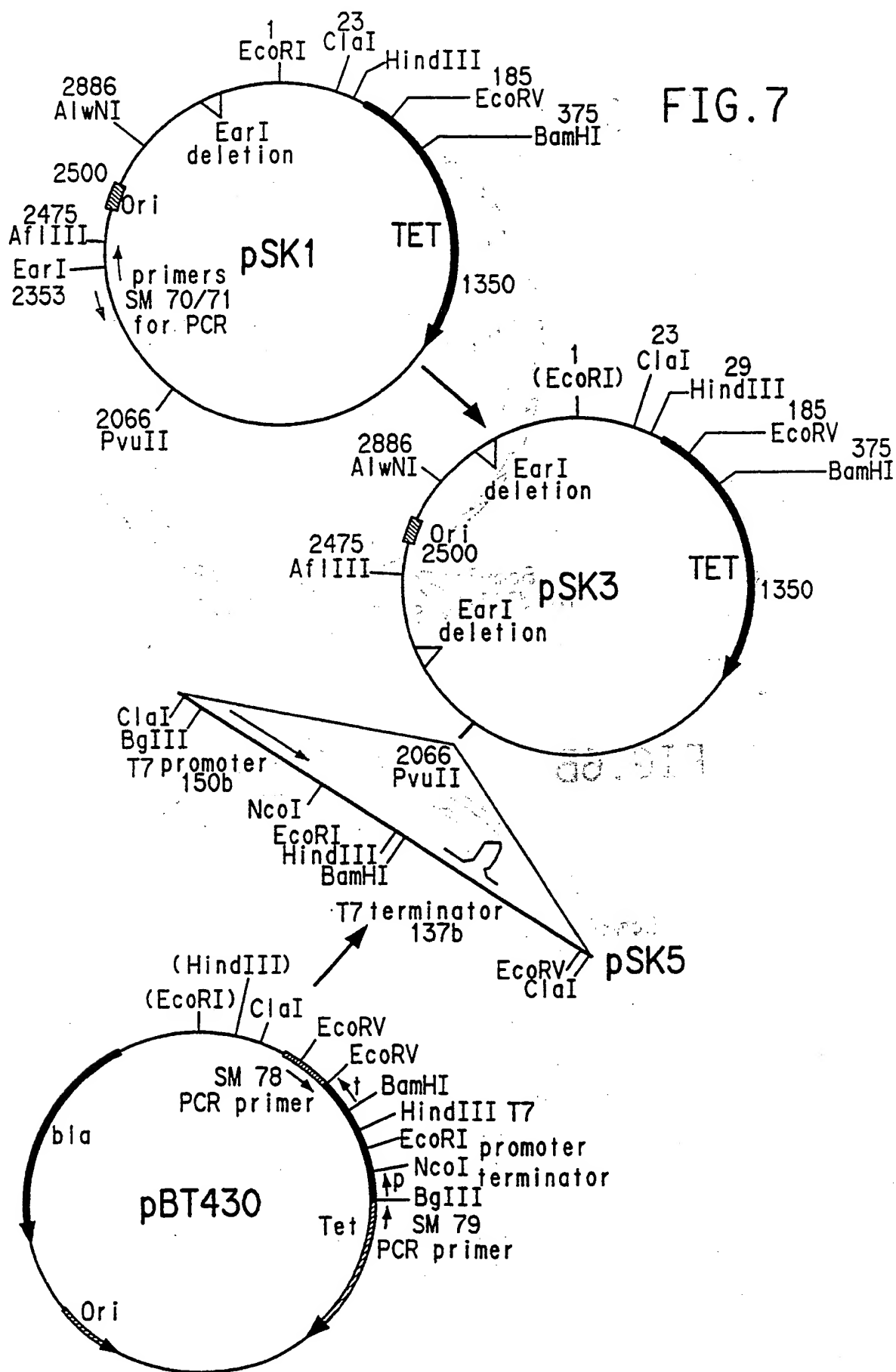


FIG. 6B



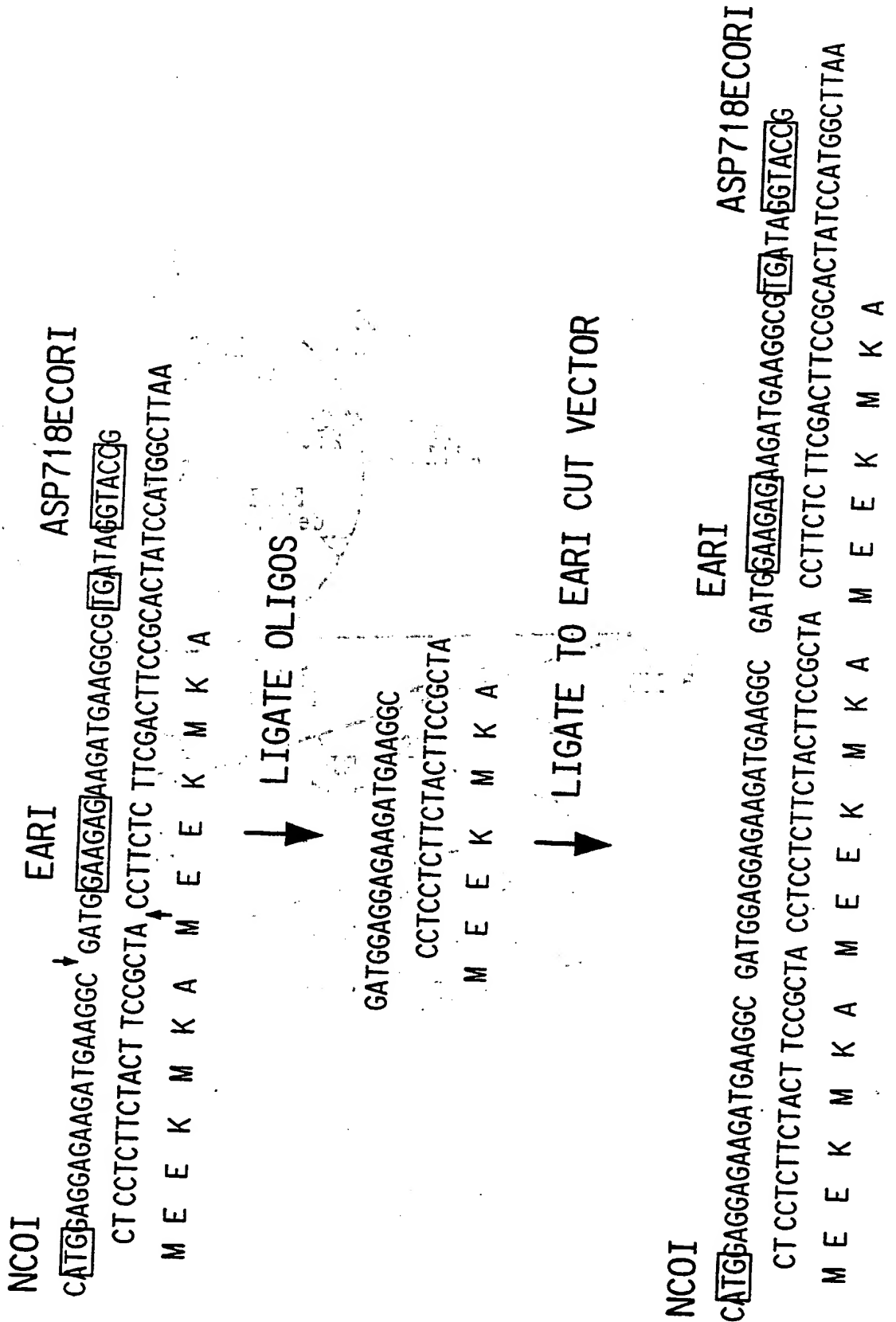
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FIG. 7



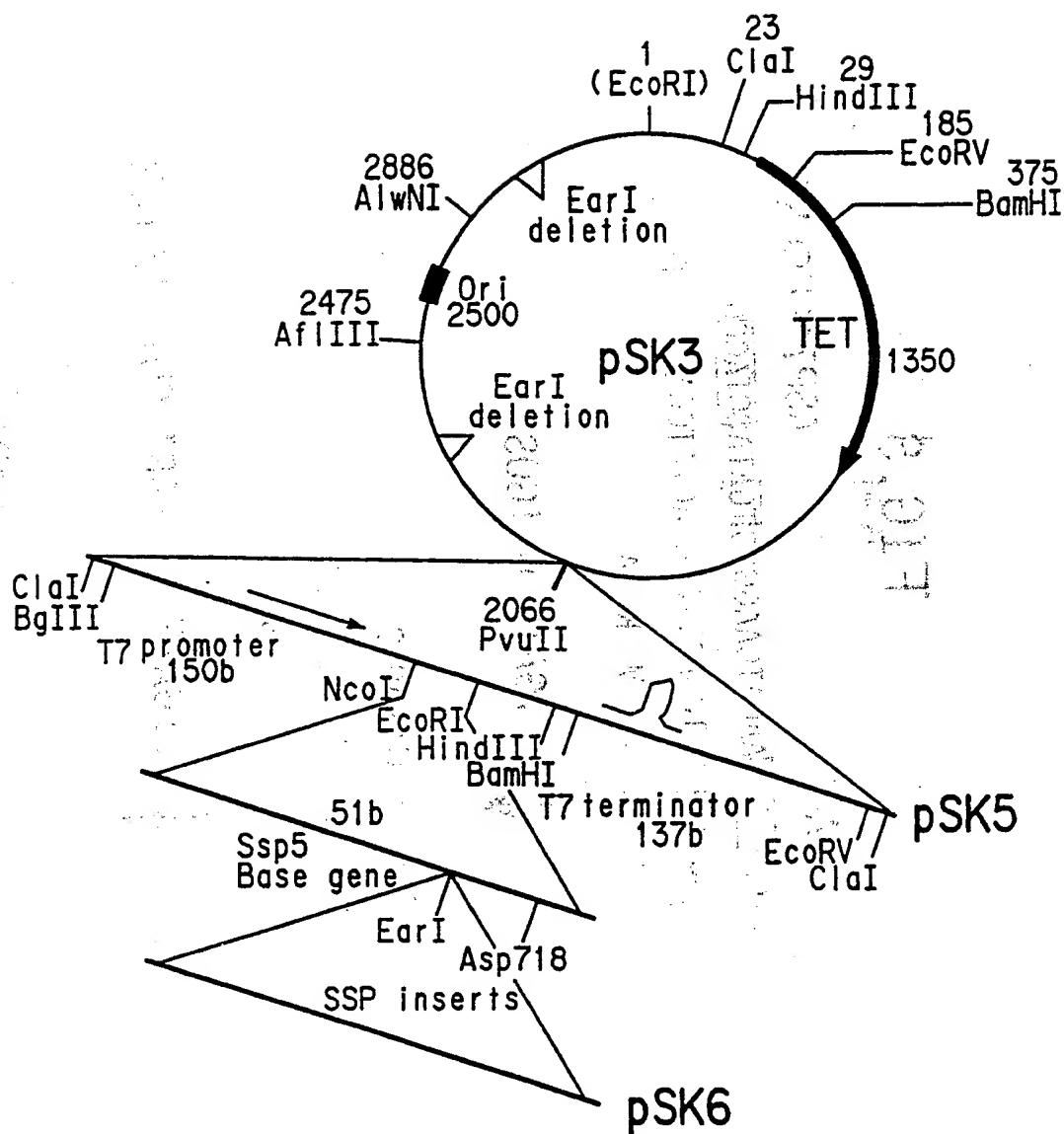
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FIG. 8



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FIG. 9





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FIG.10

NCOI CATGGAGGAGAAGATGAAAAA GCTCGAAAGAGAAGATGAAGGTCATGAAGTGATAGGTACCG ECORI STOP ASP718 ECORI BASE GENE  
 CTCCTCTTCTACTTTTCTA GCTTCTCTTCTACTTCCCAGTACTTCACTATCCATGGCTTAA  
 M E E K M K K L E E K M K V M K

OLIGONUCLEOTIDE INSERTS

GCTGGAAGAAAAGATGAAGGCTATGGAGGAGAAGATGAAATGGCTTGAGGAAAAGATGAAGAA  
 CCTTCTTTTCTACTTCCGATACCTCCTGTCTACTTTACCGAACTCCTTTCTACTTCTTCTGA  
 L E E K M K A M E E K M K W L E E K M K K

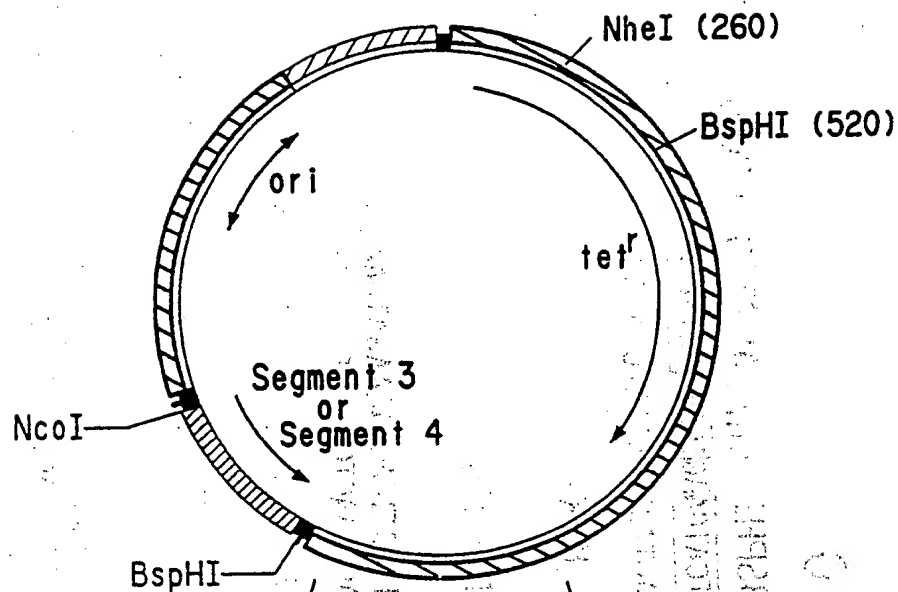
↓ OLIGOS LIGATED INTO EARI CUT BASE GENE

NCOI CATGGAGGAGAAGATGAAAAA GCTGGAAGAAAAGATGAAGGCTATGGAGGAGAAGATGAAATGGCTTGAGGAAAAGATGAAGAAAGCT  
 CTCCTCTTCTACTTTTCTA CCTTCTTTTCTACTTCCGATACCTCCTGTCTACTTTACCGAACTCCTTTCTACTTCTTCTTCTGA  
 M E E K M K K L E E K M K A M E E K M K W L E E K M K K L

EARI BSPHI ASP718 ECORI  
GAAAGAGAAGATGAAGGTCATGAAGTGATAGGTACCG  
 GCTTCTCTTCTACTTCCAGTACTTCACTATCCATGGCTTAA CLONE pSK34  
 E E K M K V M K

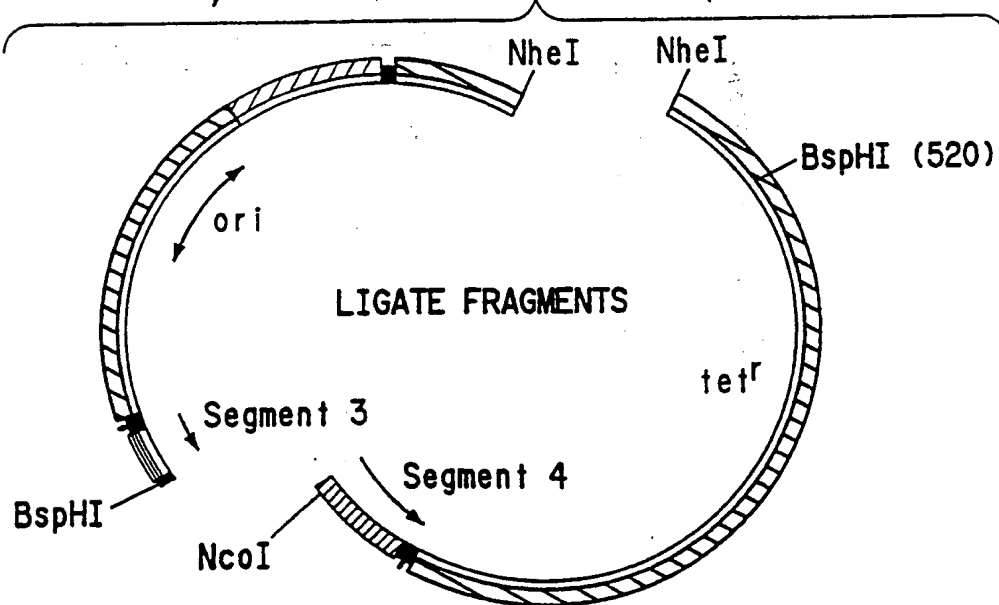
10/13

FIG. 11A



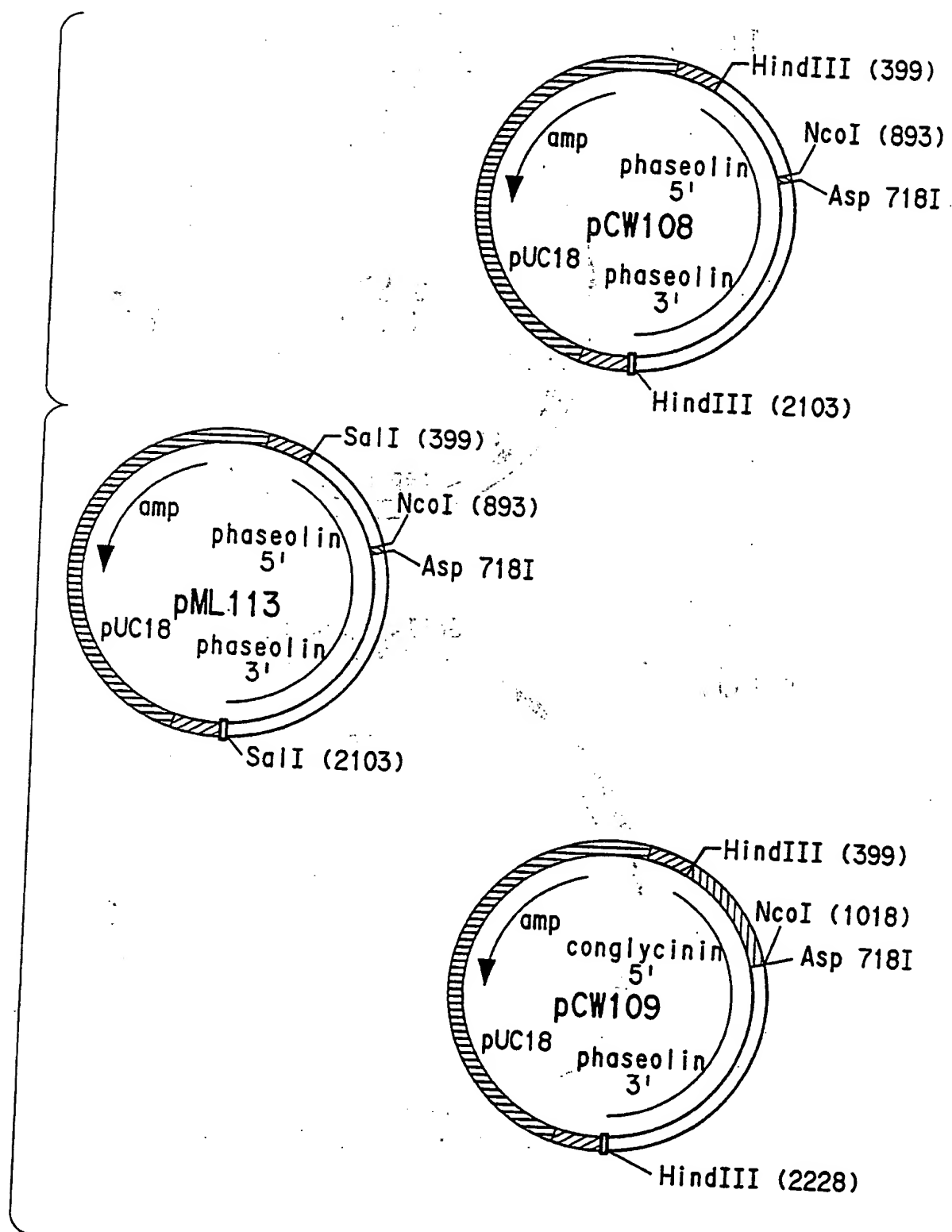
Segment 3 Digest NheI/BspHI      Segment 4 Digest NheI/NcoI

FIG. 11B



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FIG. 12



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FIG. 13

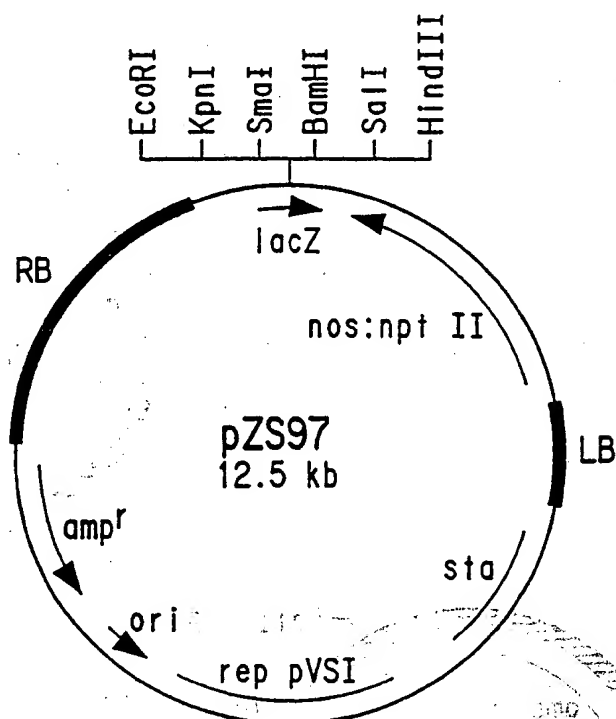
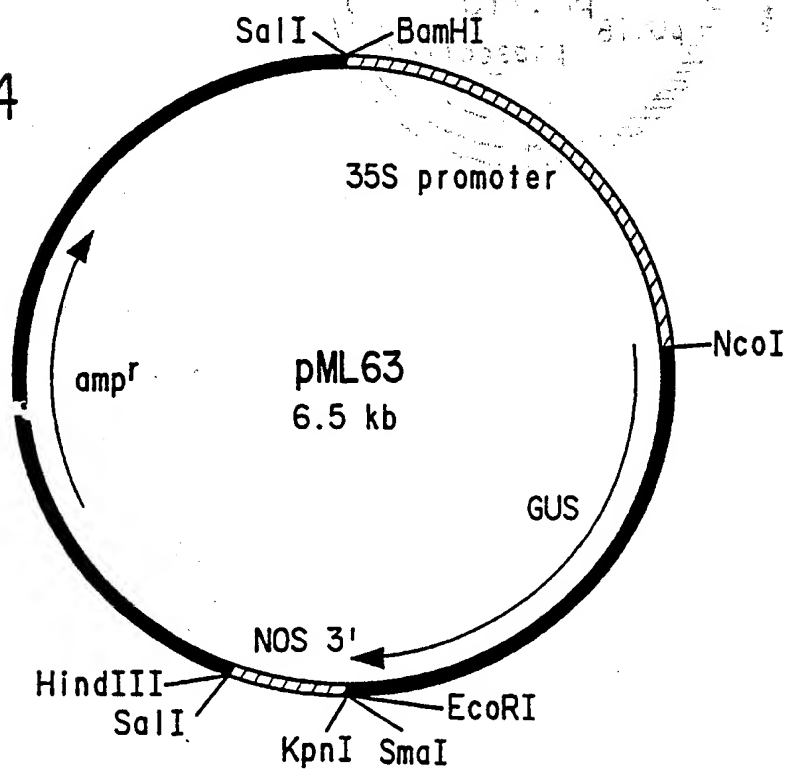


FIG. 14



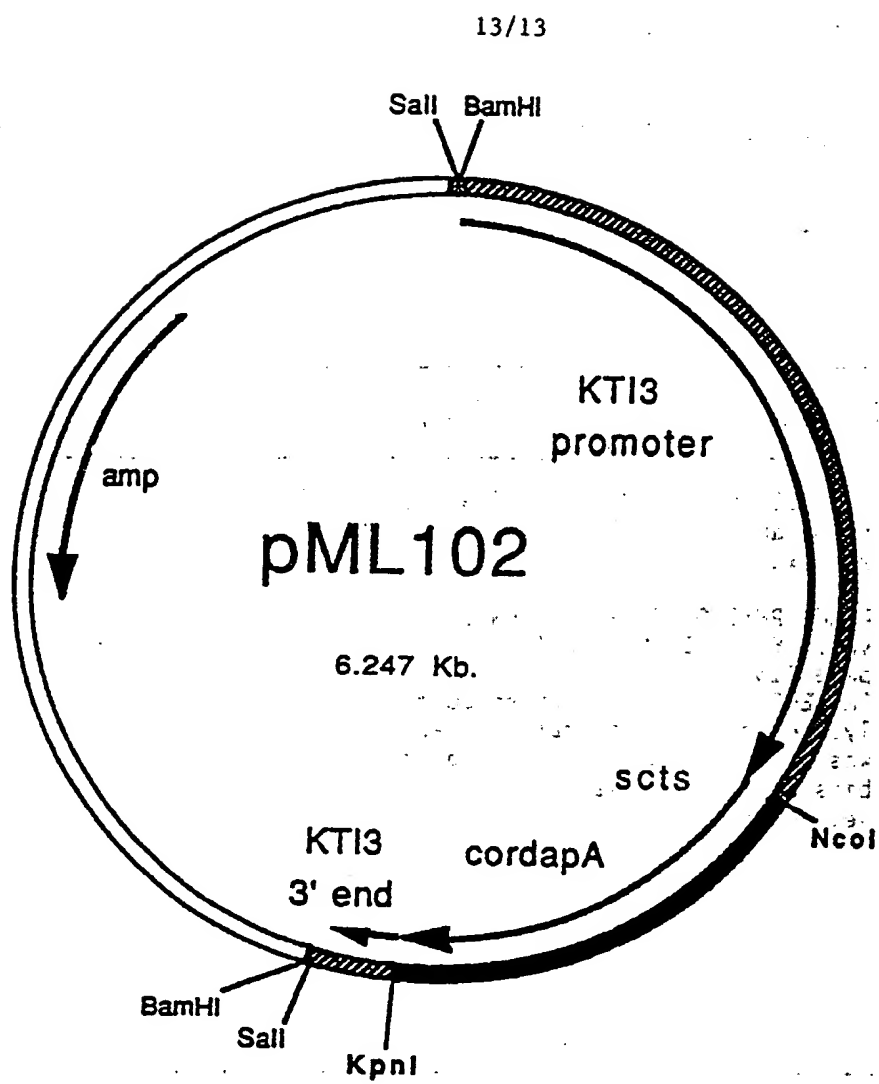


FIGURE 15

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 94/13190

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/82 C12N15/60 C12N15/11 A01H5/00 A01H5/10

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 19190 (DU PONT) 30 September 1993 see examples 3,6,10,12,15 see examples 16,19	1-6,9,10
P,X	PLANT PHYSIOLOGY SUPPLEMENT, vol.105, no.1, May 1994 page 115 SANDERS, C., ET AL. 'Overproduction of lysine in seeds of Brassica napus cv westar by genetic engineering of the biosynthetic pathway' see abstract 617	9-11

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

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\*&amp;\* document member of the same patent family

Date of the actual completion of the international search

20 March 1995

Date of mailing of the international search report

Name and mailing address of the ISA

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Authorized officer

Maddox, A

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 94/13190

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	JOURNAL OF CELLULAR BIOCHEMISTRY SUPPLEMENT, vol.18A, 1994 page 79 FALCO, S.C., ET AL. 'Transgenic crops with improved amino acid composition' see abstract X1-017 & KEYSTONE SYMPOSIUM ON IMPROVED CROP AND PLANT PRODUCTS THROUGH BIOTECHNOLOGY, KEYSTONE, COLORADO, USA, JANUARY 9-16, 1994. ---	1-5
P,A	JOURNAL OF CELLULAR BIOCHEMISTRY SUPPLEMENT, vol.18A, 1994 page 107 RICE, J.A., ET AL. 'Expression of synthetic high lysine seed storage proteins can significantly increase the accumulated levels of lysine in mature seeds of transgenic crop plants' see abstract X1-329 & KEYSTONE SYMPOSIUM ON IMPROVED CROP AND PLANT PRODUCTS THROUGH BIOTECHNOLOGY, KEYSTONE, COLORADO, USA, JANUARY 9-16, 1994. ---	21-31
A	WO,A,93 03160 (DU PONT) 18 February 1993 see the whole document -----	21-31

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.  
PCT/US 94/13190

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WO-A-9319190	30-09-93	AU-B- 3923393	21-10-93
		CA-A- 2132414	30-09-93
		EP-A- 0640141	01-03-95
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WO-A-9303160	18-02-93	AU-A- 2441292	02-03-93
		CA-A- 2114788	18-02-93
		EP-A- 0598806	01-06-94
-----			